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TISSUE MICROBIOLOGY – AN INTEGRATED APPROACH FOR INTRAVITAL STUDIES OF HOST-PATHOGEN INTERACTIONS

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By

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To Dad

The One I Aspire to Be

ABSTRACT

A bacteria infection in the living host is the summation of a myriad of interactions. Reductionistic and sacrificial experiment models cannot replicate this complexity. The Tissue Microbiology approach overcomes this by allowing the live host and pathogen to control all facets of the infection model. This enabled the discovery of many novel aspects of the pathophysiology of infection. In this thesis, we were drawn into the study of inter-organ communication networks and how they are activated during an infection. Earlier studies linked transcriptomic changes at the spleen to the kidney infection but did not show how the preceding inter-organ communication had occurred. Using the intravital infection model for experimentation and intravital imaging to guide the design of Cellular Microbiology models, we uncovered the presence of sensory afferent nerves in the renal cortex and demonstrated their unique role in sensing and communicating infections between kidney and spleen already a few hours after infection was initiated. We also showed a link between the *E. coli* toxin α-haemolysin and splenic expression of IFN-γ, which is mediated by epithelial eATP signaling. This work expands on the growing field of Nervous Driven Immunity, and demonstrated the versatility of sensory afferents in infection sensing and initiating systemic responses by swift long distance communication.

Bacteria colonizing the host microenvironment adopt a multicellular lifestyle to resist efforts of innate immunity and early-induced innate responses. While showing all indications of being a biofilm, no method exists to verify or study this *in vivo*. To overcome this we developed an optical method for continuous *in situ* analysis of biofilms. This was based on luminescent conjugated oligothiophenes (LCOs), a non-toxic small molecular fluorophore. This chameleon like oligomers allowed specific detection of *Salmonella* extracellular matrix (ECM) curli fibers and cellulose via a target specific optical signature, and enabled dynamic tracking of their formation in a variety of growth models. Named Optotracing, this technology uncovered a rare glimpse of cellulose in intracellular bacteria, in eukaryotic cells and infected tissues.

Optotracing proved to be a versatile trans-disciplinary platform and an *Enabling technology*. The unique affinity for bacteria produced cellulose was transferrable to the study of lignocellulose feedstock. p-HTEA, a pentameric cationic LCO, was highly effective for rapid, non-destructive, detection and quantification of cellulose, lignin, hemicelluloses, across materials of a variety of physical states and chemical compositions.

Optotracing, as a method for material analysis, could be easily performed on spectrophotometers and fluorescence microscopes to provide visual maps of the surface topography of lignocellulose materials. Continuous monitoring of enzymatic reactions such as cellulolysis was also possible. Investigating the mechanism of how LCOs detect cellulose revealed a 2-step process of selective binding and target specific reporting. As an example, h-FTAA selectively bond β-configured, but not α-configured glucose polysaccharide. Each β-configured glucose polysaccharide induces an identifiable target specific optical signature of h-FTAA that is determined by qualities such as saccharide length, bonding positions and projecting R-groups. This 2-step mechanism allowed for high-resolution imaging of cellulose structures in plant tissues, with virtually no cross-reactivity with the myriad of other carbohydrates present. Optotracing also allowed for high-resolution imaging and cellulose specific 'Carbohydrate anatomical mapping' of plant tissues

This thesis contributes to better understanding of host communication networks in sensing and responding to infections, and produced new technology and tools that is likely to improve the research of biofilms and cellulose materials. We envision that future development of work enclosed in this thesis will continue to produce novel tools that will accelerate the study of bacteria related diseases in humans, microbe lifestyles and plant-based materials.

LIST OF SCIENTIFIC PAPERS

I. **Choong FX**, Steiner SE, Schulz A, Bas D, Sandoval RM, Molitoris BA, Melican K, Svensson C, Richter-Dahlfors A, Nervous driven immune response during early pyelonephritis, (Manuscript 2017)

II. **Choong FX**, Bäck M, Fahlén S, Melican K, Rhen M, Nilsson KPR, Richter-Dahlfors A, Real-time optotracing of curli and cellulose in live Salmonella biofilms using luminescent oligothiophenes, *npj Biofilms and Microbiomes*, 2016, 16024

III. **Choong FX**, Bäck M, Steiner SE, Melican K, Nilsson KPR, Edlund U, Richter-Dahlfors A, Nondestructive, real-time determination and visualization of cellulose, hemicellulose and lignin by luminescent oligothiophenes, *Scientific Reports*, 2016 (6), 35578

IV. **Choong FX**, Bäck M, Schulz A, Nilsson KPR, Edlund U, Richter-Dahlfors A, Carbohydrate anatomical mapping and molecular identification of glucans using cellulose specific oligothiophenes, (Manuscript 2017)

Reviews and book chapters produced during my PhD but not included in the thesis

- **Choong FX**, Regberg J, Udekwu KI, Richter-Dahlfors A, Intravital models of infection lay the foundation for tissue microbiology. *Future Microbiol*, 2012, 7:519-533.

- **Choong FX**, Sandoval RM, Molitoris BA, Richter-Dahlfors A, Multiphoton microscopy applied for real-time intravital imaging of bacterial infections in vivo. *Methods in Enzymol*, 2012, 506:35-61.

- Melican K, **Choong FX**, Richter-Dahlfors A, Host-pathogen interactions and host defence mechanisms. In: Coffman TM, Falk RJ,Molitoris BA, Neilson EG, Schrier RW (eds). *Schrier's diseases of the kidney* 9 ed,2012, 1:673-688.

- **Choong FX**, Richter-Dahlfors A, Intravital two-photon imaging to understand bacterial infections of the mammalian host, *Methods in Molecular Biology*, 2014, 1197: 87-100

- **Choong FX**, Antypas H, Richter-Dahlfors A, Intergrated pathophysiology of pyelonephritis. *Microbiol Spectr*, 2015, 3(5): UTI-0014-2012. doi:10.1128/microbiolspec.UTI-004-2012

Patent application produced during my PhD but not included in the thesis

Granted
- **Choong FX**, Richter-Dahlfors A, Carbohydrate detection (WO2014007730 A1

Pending
- **Choong FX**, Richter-Dahlfors A, Carbohydrate detection (7 regions)
- **Choong FX**, Libberton B, Richter-Dahlfors A, Detection of microbial peptides

CONTENTS

LIST OF ABBREVIATIONS

CF	Calcofluor
CGRP	Calcitonin gene related peptide
CLSM	Confocal laser scanning microscopy
CNS	Central nervous system
CR	Congo red
CV	Crystal violet
DAMP	Damage associated molecular patterns
DAPA	Disease associated protein aggregates
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assays
Em. λmax	Wavelength of emission maxima
Ex. λmax	Wavelength of excitation maxima
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
ICN	Inter-organ communication networks
IFN-γ	Interferon-γ
IL	Interleukin
IVIS	*In vivo* imaging system
LCO	Luminescent conjugated oligothiophenes
LCP	Luminescent conjugated polythiophenes
LED	Light emitting diode
LPS	Lipopolysaccharides
M. cellulose	Microcrystalline cellulose
NF-κβ	Nuclear factor-κβ
NK	Natural killer
NOD	Nucleotide-binidng oligomerization domain
NPY	Neuropeptide Y
PAMP	Pathogen associated molecular patterns
PCT	Proximal convoluted tubule
PMT	photon multiplier tubes

PNS	Peripheral nervous system
PRR	Pattern recognition receptors
ROS	Reactive oxygen species
SCA	Salmonella containing vacuoles
SOM	Somatostatin
SP	Substance P
Th	Thioflavin
TLR	Toll-like receptors
TRPV-1	Transient receptor potential cation channel subfamily V member 1
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary tract infections
VIP	Vasoactive intestinal peptide
α7nAChR	Nicotinic acetylcholine receptor subunit α
λ_{Em}	Emission wavelength
λ_{Ex}	Excitation wavelength

1 INTRODUCTION

1.1 THE ADVENT OF TISSUE MICROBIOLOGY

On a daily basis, a healthy individual encounters vast amounts of microbes that are detected and destroyed. Where this fails, an infection develops. Broadly defined, infections occur when microbes not normally present in the body, enter and establish a foothold.

Infections are fundamentally complex. The meeting point between metabolically versatile microbes and the wide array of host barriers and responses becomes a fine balance which when tipped, can result in outcomes ranging from the asymptomatic clearance of the infection, to sepsis and mortality. This understanding stems from a small part of the vast amount of data accumulated from fields such as Microbiology, Physiology, and Histology[1]. This also includes higher order fields that have emerged over decades, that have in parallel led to the discovery of yet more possible interacting host and pathogen factors that come into play during infection[1,2].

Cellular Microbiology in particular, brought perspective into the picture using space and time to introduce relevance, filtering down the myriad of possible molecular interactions to relevant combinations that occur when host and microbes are in proximity for communication[1,2]. Drawing from Koch's postulates, this field has pioneered the development and combinatory use of clever bacterial genetics, monocultures of epithelial and/or macrophage-like cells, and detection methods to create and study interactions of greater complexity, in ways that better mimics the *in vivo* situation[3]. Extensive use of various basic tissue culture models have elegantly identified qualities such as bacterial attachment; internalization; immune response evasion; intracellular survival; and bacterial secretion systems, and what has come to be known as hallmarks of the pathophysiology of an infection[4]. Improvement of the basic tissue culture set up to include more experimental control and host-cell types, then brought about various trans-well and cell migration models which were instrumental in validating the identity and roles of cytokines and chemokines in cell-to-cell communication and immune cell recruitment[1,2]. Cellular Microbiology thus formed the benchmark approach of Infection Biology studies and became a major cornerstone of the field.

Historically, Cellular Microbiology, and much of infection research, is founded on reductionism. At the time of this writing, it is still impossible to adequately replicate biological systems in the laboratory, or fully scrutinize all collectable data of an infection within the lifetime of one academic career. It is therefore necessary to reduce an infection to

manageable research packages, where the effects of one or few participating factors or interactions are studied. Despite amazing successes and productivity in advancing the understanding of infections and identifying factors, reality proves that an infection is rarely a simple summation of the individually studied packages. Interfaces in which a pathogen and the host interact contain a heterogeneous mix of factors that often change as over time. Reductionism is unable to capture such complex events and transient factors since multi-cellularity and tissue relevance is lost in such models. Many *in vitro* findings do not translate when in animal models and in humans, and the real-life relevance of these findings are not fully understood. The challenge of Infection Biology is therefore the return to an all-inclusive research approach, whereby all condition and factors in time, of host and pathogen are taken into account.

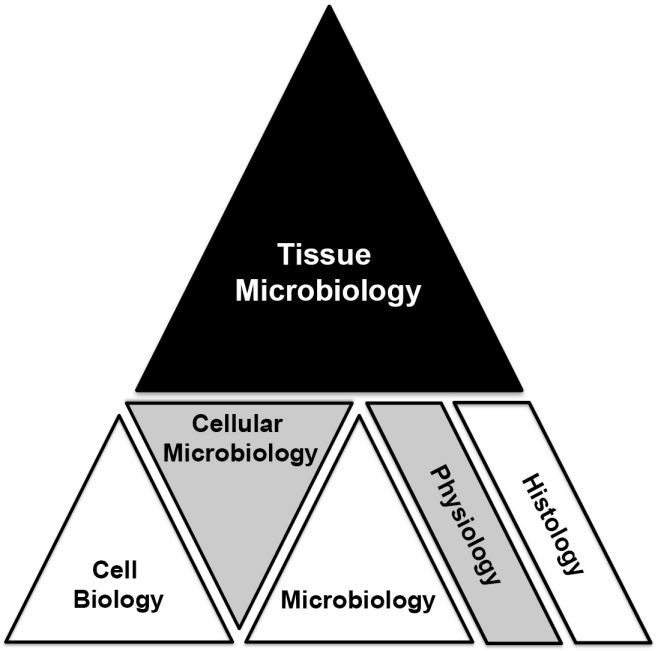

Figure 1.1: Development of Tissue Microbiology. *Our current understanding of an infection is built on the foundational disciplines of Physiology, Histology, Cell Biology and Microbiology*[5-7]*. Higher order fields such as Cellular Microbiology has sought to achieve better in vivo relevance by closer integration of the foundational disciplines. Tissue Microbiology furthers this inter-disciplinary effort by integrating Cellular Microbiology with Physiology and Histology, for an all-inclusive view of infections. Adapted from Choong et al 2012 and Richter-Dahlfors et al., 2012* [6,7].

Tissue Microbiology begins with an understanding that the outlook of an infection is the sum of innumerable known and unknown interactions between host and pathogen, and accepting reconstructions *in vitro* are often inadequate[5,7]. Tissue Microbiology traverses the gap between *in vitro* and real-life by allowing the microbe and the host to define the experiment[5-8]. This is done by allowing the tested organ to determine the experimental parameters according to the tissue's native condition and the subsequent response[5,8]. The experimentalist's role is then to monitor the infection in its entirety from all possible angles by methods available at the time[8]. Data generated by Tissue Microbiological studies is complex in both depth and range, which might span many scientific disciplines[6,7]. As such, full usage of these models can only be achieved by the collaboration of a wide range of

competencies inside and outside of Infection Biology[5-7,9]. Recognizing the difficulty of such research models, concerted effort by groups across the globe pioneering the advancement of research by Tissue Microbiology approaches have developed a wide range of techniques such as innovative surgical procedures for precise spatio-temporal control, fluorescence twophoton microscopy, physiological tissue monitoring and molecular analysis[7]. Models offering an all-inclusive perspective for the study of the pathophysiology of infection in live animals are now increasingly accessible to the research community[7].

Since its inception, Tissue Microbiology models achieved considerable success in advancing our understanding of the dynamic host micro-ecology and the pathogenic versatility of microbes[5]. Noteworthy are the efforts of the Miller group (University of California, Irvine) that demonstrated extra-vascularization and infection targeted migration patterns of immune cell types to infections and tumors in their twophoton microscopy studies of *Listeria monocytogenes*[10]; the IVIS (*In vivo* imaging system) based studies of Mobley group (University of Michigan) showing the flagella and fimbria mediated ascending colonization of uropathogenic *Escherichia coli* (*E. coli,* UPEC) during urinary tract infection[11,12]; the twophoton microscopy and transcriptomics based animal studies of the Richter-Dahlfors group (Karolinska Institutet) showing the involvement of microbial fitness factors, the coagulation cascade and inter-organ communication in determining infection dynamics[8,13-15]; and the animal work of the Tracey group (Hofstra Northwell School of Medicine) elucidating nerve based inter-organ communication of infection to the spleen, and host protection by the controlled inflammation[16-26]. Hereinto, such Tissue Microbiological models that utilize animals as platforms will be referred to interchangeably as intravital studies.

1.2 THE TISSUE MICROBIOLOGY VIEW OF PYELONEPHRITIS

Listed as one of the most common infections, urinary tract infections (UTI) account for a quarter of hospital acquired infections with a mortality rate of 2.3%, and approximately 1.6 billion USD in healthcare cost in the United States of America[27-29]. Attributed mostly to *E. coli* (80%)[27-29], UTIs occur when bacteria enter the urinary tract through the urethra. Initial colonization of the bladder by bacteria, known as cystitis, can exacerbate to pyelonephritis when bacteria ascend urinary tract to infect the kidneys, and subsequently disseminate systemically to cause urosepsis, which accounts for 5 % of all sepsis cases[30-37].

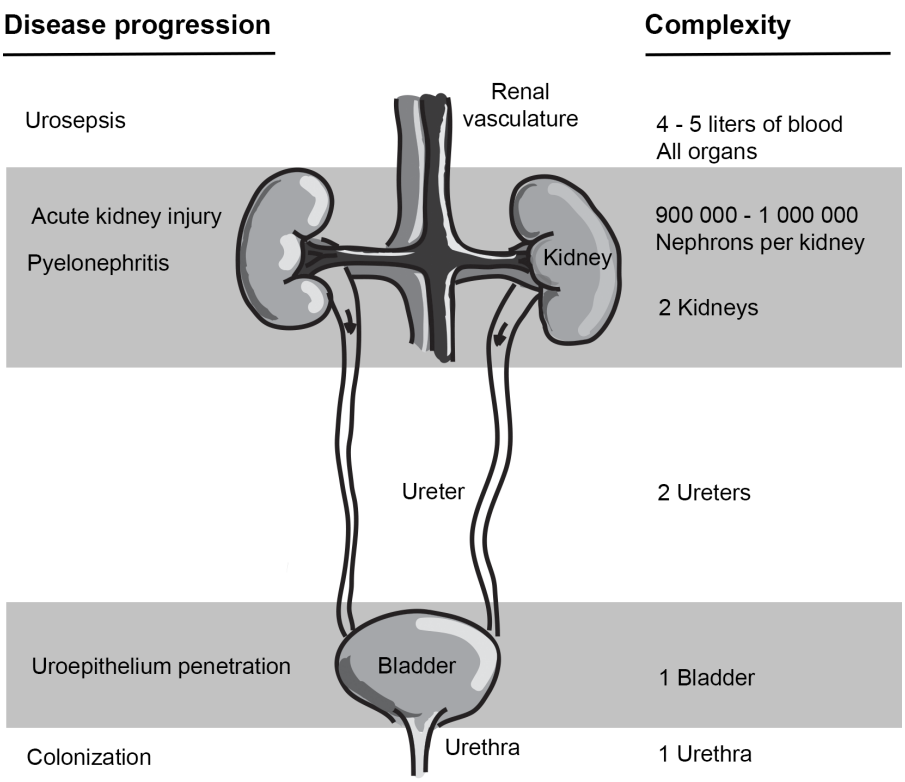

Figure 1.2: Microenvironments of UTI their complexity. *Bacteria that enter the urinary tract colonize and survive this challenging microenvironment by a range of adhesion, fitness and virulence factors*[38]*. With distinct variations between organs of the urinary tract, bacteria are compelled to field different factors in each microenvironment*[38]*. Molecular switching of expressed factors is also necessary when bacteria transition between organs when ascending the urinary tract*[38,39]*. Reproducing this complexity in vitro is exceedingly difficult. Tissue microbiology solves this by using the organ itself as the 'test-tube', allowing the host to dictate the conditions of experimentation*[5]*.*

Tissue Microbiology work pioneered by the Richter-Dahlfors group focused on the nephron, taking advantage of the close association of epithelial, endothelia and connective tissue layers unique to this organelle, to study host–pathogen interactions during pyelonephritis[8]. Using precision surgery, the kidney of a live anesthetized rat is carefully exposed for access to one surface located proximal convoluted tubule (PCT) among the millions of nephrons present[5,6,8]. Microinjection with a custom made needle, pulled to a tip with an inner diameter of 4-7 μm, allowed the delivery of bacteria into the lumen of a single proximal tubule, at a precise and defined time-point[5,8,40,41]. The ensuing infection is then cinematically recorded in one of the best-known spatio-temporally platforms for the study of infection[5,8,40,41].

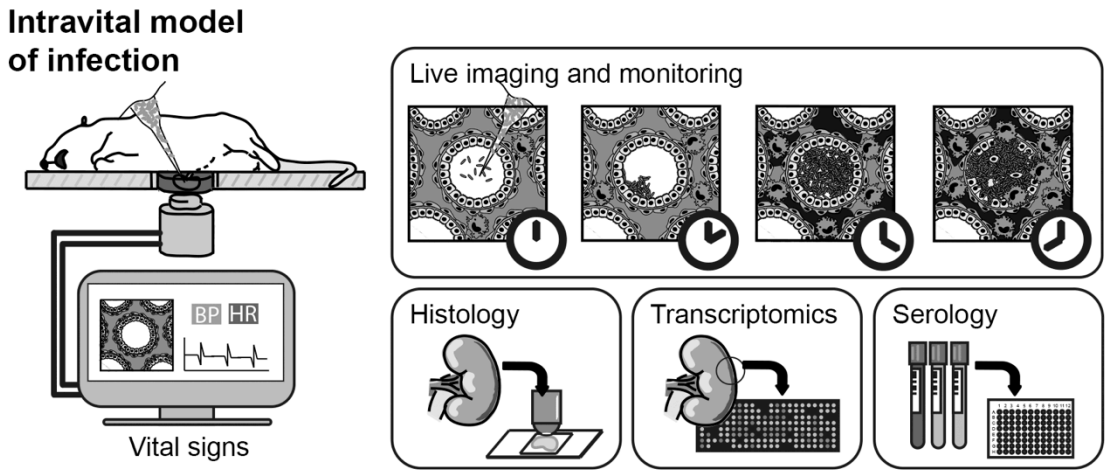

Figure 1.3: Multifaceted analytics enabled by the intravital model. *The intravital model of infection simulates pyelonephritis by direct injection of bacteria into a single nephron of the kidney of a live anesthetized rat*[8]*. Ensuing changes in host vital signs can be recorded simultaneously. Positioning the infected kidney over a microscope objective allows for fluorescence based live imaging and monitoring of host-pathogen interactions, histological and physiological changes of fluorophore and auto-fluorescing tissue compartments. High spatio-temporal resolution of the infected tissue allows for the key time points in host pathogen interactions to be examined further via immunofluorescence and immunohistochemistry, transcriptomics and serology*[8,13-15]*.*

Using fluorescence twophoton microscopy, Månsson *et al*., 2007 first described the intravital model of UPEC infection of the PCT and beautifully showed the protective role of hydrodynamic pressure, as well as the resilience and determination of UPEC to infect[8]. The work also showed the effectiveness of the host in clearing the infection. UPEC injected into a renal tubule is immediately subjected to the vicious sheer stress arising from glomerular filtration[8]. This eliminates a large population of the bacteria[8]. The few remaining bacteria that are retained through adhesion to the tubular epithelium rapidly adapt to the microenvironment, after which colonization occurs with such voracity that the luminal space is completely filled by bacteria within hours[8]. Extending the study over 24 h, Månsson *et al*., 2007 was able to glimpse the conclusion of UPEC infection[8]. Local and controlled tissue destruction with the massive influx of immune cells resulted in a deep void in fluorescence, in which neither nephron nor bacteria remain [8]. Meanwhile, adjacent nephrons were found to be unaffected and functioning normally[8].

Subsequent work by Melican *et al*., 2008 furthered what we know about the host response[14]. Early into the infection, the tissue experiences several alterations in histology and physiology[8,14]. Upon colonization by UPEC, the epithelium breaks down is sheds with the luminal flow[14,42,43]. This reduces UPEC load by eliminating established infections, and reducing epithelial attachment sites available. In parallel, vascular coagulation occurs at the

endothelial face and develops into vascular shutdown (within 5-6 h of infection), limiting the dissemination of bacteria from the infection site[14,44]. The secondary effect of this physiological change is the dramatic drop in local tissue oxygen[14]. This delimitation of the primary infection site is thought to buy time for the recruitment of the polymorphonuclear cells and other host cellular immune responses[8,14,45]. In the meantime, the release of antimicrobial/cytotoxic molecules causes the destruction of the infection as well as the host tissue (edema and necrosis)[46-48]. The combined effect of this rapid and focused host response is the highly efficient clearance of the infection (within 22 h) similarly reported earlier by Månsson *et al*., 2007[8,14,49].

Turning attention to the pathogen, Melican *et al*., 2011 revealed the intricate role of UPEC fitness factors in surviving and colonizing despite challenges of the renal microenvironment, by combining the intravital model of UPEC infection with genetic techniques[15]. Opposition of filtrate flow in the nephron occurs by P-fimbriae mediated attachment to glycosphingolipids present on the uroepithelium via the tip adhesin PapG[15,50,51]. PapG mediated attachment allows bacteria to propagate two-dimensionally along the luminal face of the PCT, forming a hollow tube on uroepithelium when in the absence of inter-bacterial attachment factors[15,50-52]. Melican *et al*., 2011 then showed that the expression of Type-1 fimbriae and the FimH adhesin was essential for propagation into the 3rd dimension by allowing UPEC to resist filtrate flow and fill the luminal space of the PCT[15,53-56]. This work showed that P-fimbriae, while enhancing infection, was not essential for the establishment of infection[15,57]. Absence of PapG adhesins merely changed the dynamics of which infection was achieved. UPEC lacking functional PapG adhesins retained in the PCT by forming large Type-1 fimbriae mediated intra-luminal bacterial clusters that greatly resisted clearance by filtrate flow[15].

Alongside Melican *et al*., 2011, Boekel *et al*., 2011 applied the unprecedented spatio-temporal control of the intravital model to enhance transcriptomics analysis of UPEC infections, and produced a global view of the multifaceted infection through a Systems Biology perspective[13]. Comparison of transcriptional profiles of UPEC-infected biopsies with adjacent uninfected tissues within the same kidney showed distinct differences in expressed genes as early as 3 h, gaining statistical significance as the infection progressed[13]. At 8 h, 59 differentially expressed genes were observed, among which 25% were identified to be interferon-γ (IFN-γ) regulated[13]. IFN-γ modulates immune and inflammatory responses, ensuring controlled bacterial clearance while limiting inflammation and tissue damage[13,58,59]. The work hypothesized an infection induced inter-organ network of communication whereby

interleukin (IL)-23 from the infected kidney induces a systemic increase in splenic IFN-γ, directly or indirectly via IL-17[13,58,59].

Collectively, the intravital model of UPEC infection achieved new insights of the integrated pathophysiology of infection from the perspective of both microbes and host[8,13-15]. The model also proved to be a versatile platform that could easily be integrated with genetics and molecular, immunohistochemical and optical methods, to study highly unpredictable host-pathogen interactions[5,7].

1.3 INTER-ORGAN COMMUNICATION

The human body is made up of approximately 37 trillion cells organized into individual and identifiable functional units of specialized cells, tissues and organs working in unison[60]. This synchronicity is possible due to the development of long distance systems of communication between functional units, or inter-organ communication networks (ICN)[61,62]. ICNs are systems of cytokines, chemokines, metabolites, electrical signals and cells that act between organ systems to communicate and coordinate signals and responses under homeostasis and stress events[62-66].

The hemodynamic system is perhaps the oldest and best studied ICN[67]. The primary infection site is rich in pathogen associated molecular patterns (PAMPs), present on the surface of bacterium[46]. Soluble PAMPs may also be shed passively, actively, or when bacteria are damaged[68,69]. Host cells in the vicinity efficiently pick up these potent stimulators of the host response by an array of highly sensitive pattern recognition receptors (PRRs), and respond by secreting a range of cytokines and chemokines[46,70-72]. Host cells that are damaged by either pathogen or inflammation also release damage associated molecular patterns (DAMP)[46,72,73]. Collectively, signals from both host and pathogen diffuse from the infection foci into circulation and signal for the recruitment of various waves of circulating immune cells (macrophages, neutrophils, dendritic cells, natural killer cells) to control and clear the infection[46,70,72]. Signals may also travel large distances and alert distal organs of the infection, which have been known to respond with cytokines that modulate immune responses at the primary infection site[62,64-67].

The nervous system links all cells of a multicellular organism by communicating information of the external and internal environment, and by coordinating actions of each body part[74,75]. The nervous system is composed of two cell types, neurons that transmit signals, and glial cells that serve to support them[74]. In humans and other vertebrates, neurons and glial cells are organized into the central nervous system (CNS), consisting of the brain

and spinal cord, and the peripheral nervous system (PNS)[74,76]. The PNS connects the brain and spinal cord to all parts of the body[74,76]. The PNS is an immense network of sensory and motor nerves that encompasses the somatic nervous system, the autonomic nervous system, the sympathetic and parasympathetic networks, and the enteric nervous system[74,76]. In the turn of the century, the nervous system gained spotlight in the seemingly unrelated field of Infection Biology. This originates from the discovery of an abundance of common and overlapping ligands and receptors between the nervous and immune systems that indicates the possibility of two-way communication and regulation[16,77,78]. Bi-directional communication between the nervous and immune systems has been observed as early as 1980s, by the concert of groups working in the field of Neuro-Immunology[79].

Immune to Nerve: Early work studying the nervous and immune systems observed significant alteration of behavioral and physiological activities controlled by the CNS upon administration of interleukins (IL) at peripheral sites of the body[80-82]. Circulating ILs were found to pass actively across the blood brain barrier through specific and saturable transport systems to exert effect on the CNS[79,83-85]. This was game changing as the CNS is often thought to be a pristine tissue compartment, made exclusive by the blood brain barrier that blocks the entry of all but a few select nutrients and neurotransmitters[84,86,87]. Receptors for a range of cytokines have since been identified in various brain areas, such as the hypothalamus, hippocampus and the pituitary[88-93].

In the periphery, bacterial lipopolysaccharides (LPS) and various cytokines communicate with the nervous system through the vagus nerve by activating sensory afferents[94,95]. Several groups found that infection related changes in the CNS controlled activities induced by ILs and LPS, could be inhibited by transecting the vagus nerve below the diaphragm[94,96-98]. It is also thought that immune activation of the vagus nerves can occur at the nodose ganglion, where Toll-like receptor (TLR) 4 expressing neuronal cell bodies could be activated by LPS should it reach this compartment[94]. Subsequently, it became apparent that the nervous system is highly receptive to signals of the immune system with the discovery of yet more cytokine and PAMP receptors on neuronal cells[25,93,95,99].

Nerve to Immune: The same neurotransmitters and neuroendocrine factors that mediate signal transmission in the nervous system were found to modulate components of the immune system. In one pathway, the sympathetic pathway releases catecholamines via noradrenergic sympathetic nerve fibers to lymphocytes at the spleen[88,100]. One effect of this is the increase of splenic neutrophils and peritoneal machrophages[101]. At lymphoid organs, catecholamines interact with β-adrenoceptors to enhance cell proliferation, migration, the production of

cytokine and antibodies, and other immune cell activities[88,89,100]. Among tested catecholamine pathways, this modulation of epinephrine and/or β-adrenergic signaling is implicated in controlling systemic inflammation during sepsis, via α- and β-adrenergic pathways[102-104]. Abrogation of catecholamine signaling resulted in the inhibition of lymphocyte proliferation in the spleen and blood[105]. Furthermore oxidopamine destruction of noradrenergic neurons at the periphery ceased production and expression of splenic IL-2 and IFN-γ[105]. At the CNS, this significantly reduced natural killer and T-cytotoxic cells in spleen and peripheral circulation[106].

Dopamine, a catecholamine neurotransmitter, has also been shown to communicate between nervous immune pathways[93]. Deleplanque *et al*., 1994 showed that abrogation of mesolimbic dopaminergic networks by oxidopamine destruction of dopaminergic projections in the mesolimbic nucleus accumbens depressed the splenic and NK cell proliferation and activity[107]. Conversely, administration of dopamine at the spleen enhanced lymphocytes proliferation, and additionally inhibited the production of splenic IFN-γ[108]. Further work by Torres *et al*., 2005, showed the presence of increased production of cytokines of both pro- and anti-inflammatory pathways in peripheral blood mononuclear cells stimulated with dopamine and norepinephrine[109].

Finally, neuropeptides are also potent communicators between the nervous and immune system. Vasoactive intestinal peptide (VIP) for example, inhibits the expression of pro-inflammatory cytokines in macrophages, by interfering with the activity of transcription factors[110-112]. VIP also promotes the proliferation of certain T-cell subtypes. To date, calcitonin gene related peptide (CGRP), neuropeptide Y (NPY), somatostatin (SOM), and substance P (SP) have also been shown to have direct effect on the immune system[113-115].

The inflammatory reflex: The inflammatory reflex describes an integrated neural circuit that regulates inflammatory responses through a series of nervous-immune communications[16-18,116]. A well-known effect of this circuit is the prevention of immune mediated damage by controlling the production of pro-inflammatory cytokines[18-21,117,118]. Activation of this circuit begins with the binding of PAMPs or DAMPs released during infection or injury to various TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors and other PRRs on cells[99,117-121]. Stimulation of PRRs sets off a cascade of signals within cells that converge on the activation of nuclear factor-κβ (NF-κβ)[120,121]. NF-κβ initiates the transcription and production of a myriad of pro-inflammatory cytokines that activates innate immune responses[120,121]. Additionally, cytokines released into the extracellular milieu activate afferent sensory neurons in the vicinity, thereby translating the

initial short distance proteinaceous signal into far reaching electrical signals that is relayed via the vagus nerve and organized at the brain stem[18,19,23,25,119]. In the cholinergic anti-inflammatory pathway, signals travel through the efferent arc of the vagus nerve to the celiac ganglion where they are channeled through the splenic nerve to the spleen[77]. Efferent projections at the spleen releases norepinephrine that activates T-cell populations, that in turn releases acetylcholine that activates macrophage populations via surface localized nicotinic acetylcholine receptor subunit α (α7nAChR)[17,26,116,120,121]. Ultimately, acetylcholine stimulation of splenic macrophages suppresses NF-κβ activation and reduces the release of pro-inflammatory cytokines and prevents excessive-inflammation of the immune system[77,99,120,121].

1.4 MULTICELLULAR BACTERIAL LIFESTYLES

UPEC colonization of the urinary tract is highly multicellular and resembles a type of bacterial lifestyle commonly termed 'biofilm'. Biofilms are naturally formed multicellular microbial communities enclosed in a myriad of matrix materials formed as part of the bacteria's mode of growth[122-131]. Despite their presence in fossils dating back to ~3.25 billions years ago, biofilms were first described in Zoebell *et al*, 1943, and has since gained recognition as an important factor in microbe related diseases[132,133]. This comes as a result of strong concerted efforts across the globe, as well as the advent of high-resolution three-dimensional imaging tool, techniques and reporters[134].

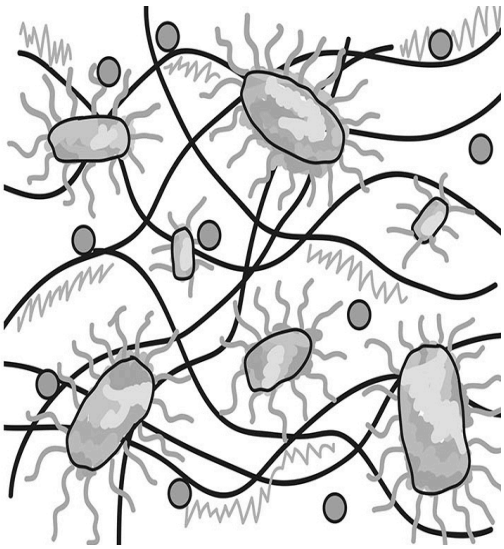

Figure 1.4: The composition of biofilms. *Biofilms are dense assemblies of bacteria and extracellular materials heterogeneously organized into a matrix. It is thought that bacteria constitute only 10% of the overall dry biomass[125]. Known extracellular materials include extracellular DNA, fimbria, pili, functional amyloid proteins, polysaccharides and lipopolysaccharides[122,125,135]. The identity and combination of extracellular materials confer bacteria a multitude of abilities and functions and can be vastly different between bacteria and a bacteria's lifecycle.*

The biofilm lifestyle can be described by a 5 stage cycle that starts from (I) the attachment of bacteria to surfaces[124,126,132,136,137]. This occurs via electrostatic and hydrophobic interactions between an array of extracellular matrix (ECM) materials such as extracellular proteins (e.g. flagella, pili, fimbriae and functional amyloid proteins),

polysaccharides (e.g. DNA, alginate and lipopolysaccharides) and lipids, with the surface[125,126]. (II) Subsequently, the initial reversible interaction with the surface is reinforced by increased presence of extracellular materials, transitioning to a strong irreversible mode of attachment[126,127]. (III) Attached cells divide and secrete ECM materials, forming micro-colonies that are become increasingly resistant to the extracellular environment[125,138-140]. The composition of the ECM may be multifarious and unique to each microbe and growth conditions, the synthesis of which is controlled by quorum sensing and other bacterial signaling factors[125-127]. (IV) Continued replication and ECM production then turns the micro-colony into a mature biofilm wherein bacteria are now encased by a dense system of barriers and channels formed by the ECM[126,127]. (V) The mature biofilm now becomes a node that disseminates bacterial into the environment. Bacterium that spread in this manner may reabsorb onto adjacent surfaces thereby seeding the formation of new biofilm communities[126,127].

Biofilm infections may develop from established single bacterium that have penetrated host tissue barriers, or from the exposure to preformed biofilms on implants, catheters and other patient critical surfaces[133]. Both modes are highly relevant to UTI by UPEC described in preceding sections of this thesis. Biofilms are detrimental for several reasons. First, in concert with bacterial adhesion factors, ECM materials are rich in hydrophobic motifs that enable bacteria to attach and colonize almost all biotic and abiotic surfaces[133,137,141-144]. Second, the ECM is thought to trap and neutralize molecular antimicrobial agents such as reactive oxygen species (ROS) and antibiotics, protecting encased bacteria from applied treatments[133,141]. Third, populations of bacteria within the biofilm enter a state of dormancy and become resistant to certain antimicrobials, particularly β-lactams[133]. Furthermore (fourth), the ECM may be organized into micro-channels and reservoirs, allowing the accumulation and exchange of nutrients and essential growth factors/signals[125,138,145-147]. Biofilms are thereby able to persist over long period and harsh conditions.

1.5 THE BIOFILM TOOLBOX

Existing methods for the study of biofilms revolve around the use of dyes in colorimetric assays and microscopy[148,149]. This section describes the state of art, broadly classified by what they bind and the method of use.

Universal stains: Perhaps one of the most common colorimetric method, crystal violet (CV) indiscriminately stains all components of the biofilm. The resultant violet ‘cloud’ following the removal of excess dye provides an approximation of the biomass present[149,150].

Protein stains: Thioflavin (Th) derivatives and Congo red (CR) are common dyes used in the study of amyloid diseases such as Alzheimer's[151,152]. Both are semi-specific for amyloid protein aggregates and are often applied as an end-point staining solution to quantify amyloid proteins in a biofilm[153,154]. CR in particular is used as an additive in solid growth medium that is differentially taken up by extracellular amyloid curli fibers and cellulose fibers as they are formed[154]. The visual appearance, or morphotype, is then an indication of the composition of the biofilm colony[154].

Polysaccharide stains: Calcofluor (CF) and the Hoechst family of molecular probes are fluorescent dyes used to study biofilms that possess polysaccharides in the ECM[155,156]. CF is a semi-specific fluorophore that binds cellulose as well as a number other polysaccharides[155,156]. Chitin, a major component of fungi cell walls is one such example[157]. CF can be applied as an additive in solid growth medium that integrates into ECM cellulose as it forms, as well as in endpoint staining of biofilm samples for microscopic analysis. The Hoechst family of molecular probes is used to image the nucleus when studying cells under microscopy. In *Bacillus subtilis* and *Enterococcus faecalis*, which utilizes extracellular DNA as a component of the ECM, the Hoechst family of molecular probes are highly effective biofilm dyes[158,159].

Cell tracking: It is commonplace to approximate a biofilm's biomass by fluorometric tracking of the cellular component present. Studies commonly use a cocktail SYTO® 9 and propidium iodide stains, selectively staining live and dead microbial cells encased in the biofilm ECM[160]. Inducing the production of fluorescent proteins is another method for tracking biofilms by their cellular component. Bacteria that can be cultivated in the laboratory are transformed with plasmids or gene segments harboring fluorophore encoding genes[161]. Bacteria expressing fluorescent proteins present in a biofilm are then detected by spectroscopy and fluorescence microscopy.

I**mmunofluorescence**: Antibodies are one of the mainstay detection tools in research to date. Used directly (fluorophore linked primary antibodies) or indirectly (fluorophore linked secondary antibodies), antibodies allows for the quantitative and qualitative analysis of biofilms, via enzyme-linked immunosorbent assays (ELISA), and fluorescence microscopy. Despite advantages of high target specificity based on the epitope used to raise it, antibody use is not commonplace. Most antibodies for biofilm studies are generated in-house by focus groups and are not commercially available. Well-known examples are antibodies for curli and fimbriae[162,163].

A critical part of current colorimetric and microscopy biofilm analysis, prior to work done in this thesis, is the removal of unbound or excess dyes and antibodies[148]. Often, this comes in the form of a washing step in the staining protocol[148]. Physical removal of applied dyes inadvertently applies a bias towards well-attached biofilm phenotypes[148]. Along with discarded washing solutions, planktonic cells, as well as a good proportion of unattached and weakly attached biofilms are lost[148]. This likely leads to an underestimation of biofilms during experimentation.

1.6 FLUORESCENCE REPORTERS AND IMAGING SYSTEMS

Fluorophores are molecules that absorb and emit fluorescent light[164,165]. Electrons within a fluorophore absorbs energy within the fluorophore's absorption spectrum which elevates electrons from the ground state to an excited state[164-166]. The return of electrons to their ground state often involves the emission of energy in the form of fluorescence, in a process known as radiative transition[165]. To date, a massive library of fluorophores has been developed[164]. Each fluorophore has a unique excitation wavelength, Stokes shift and emission wavelength. Fluorophores also range in size, from molecular fluorophores (0.4 nm) to quantum dots (5-20 nm)[164,165,167].

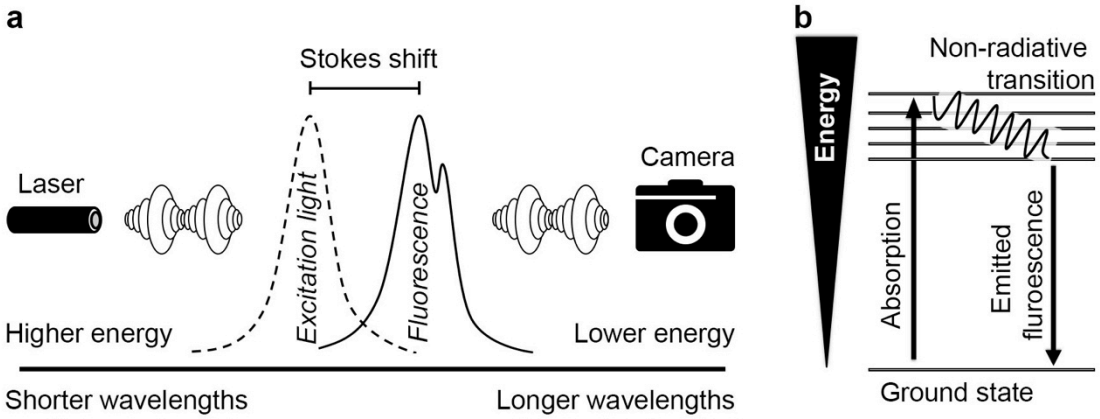

Figure 1.5: The mechanics of fluorescence, spectrophotometry and imaging. *(a) Fluorophores when used to analyze biological samples receive energy via a beam of light supplied from a laser or LED[164-166]. Fluorophores absorb energy within a specific range of wavelengths. Emitted fluorescence is collected by a camera or PMT[164-166]. The difference between the maxima of excitation and emission spectra is the Stokes shift. (**b**) The Jablonski diagram. Energy from an excitation source elevates a fluorophore to a state of higher energy from its ground state[165]. At this point, a small amount of energy is lost by non-radiative transition, the degree of which accounts for the magnitude of the Stokes shift[165]. The remaining energy emitted as the fluorophore returns to ground states constitutes the detected fluorescence[165].*

Fluorescent reporters are composed of a fluorophore and a targeting mechanism. Often, this targeting mechanism is an antibody (11 nm) that is directly linked, or a combination of a primary and a secondary antibody[167]. This combination of fluorophore and antibodies often results in a fluorescent reporter that is quite significant in size. Targeting of fluorophores can also be achieved by genetic techniques[161,164]. Transformation of bacteria with gene constructs that encode fluorescence proteins, have been used to label cellular proteins and illuminate cellular compartments[161,164].

Confocal laser scanning microscopy (CLSM) is an optical imaging technique used extensively in biology and medicine, attributed to Marvin Minsky who demonstrated the use of a prototype in 1955[168,169]. In CLSM, a specimen positioned on a microscope stage is excited by light (laser)[166,169]. Fluorescence emitted by fluorophores within the specimen are collected by photon multiplier tubes (PMTs) through a series of dichroic mirrors, excitation and emission filters[166]. An enhancement over base fluorescence microscopy systems, CLSM allows the analysis of specimens by optical sectioning, a process by which a specimen is analyzed sequentially at different depths and reconstructed into highly informative three dimensional fluorescence micrographs[161,166,170]. This is possible due to the presence of a pinhole aperture positioned before the PMT that narrows down all fluorescence signals to that which originated from a single point of focus[166,168,169]. Popularly referred to as point scanning, the pinhole aperture also serves to improve optical resolution by excluding out-of-focus fluorescence[166,169]. However, the higher optical resolution on a CLSM comes at a price. Presence of the pinhole aperture blocks a significant proportion of in-focus fluorescence from the specimen. As such, CLSM analysis often requires longer periods of laser exposures or the use of greater laser power. Prolonged laser exposure often leads to the permanent degradation of fluorophores, a process known as photobleaching. This complicates the analysis, and is a core concern when selecting fluorophores for use, and designing CLSM strategies.

Spectrophotometry is a quantitative study of the absorption and emission of photons by a specimen[165,166]. White light from a laser or LED is focused by a collimator (lens) and split into the constituent wavelengths by a prism[165,166]. Specific wavelengths of light are then selected by a monochromator and projected through a sample and collected by a PMT[165,166]. Often, wavelengths in the ultraviolet (185–400 nm) and visible range (400–800) are used. In basic applications of spectrophotometry, the concentration of molecules is estimated by the quantum of photons absorbed (optical density), or the photons emitted (fluorescence and luminescence). Recent spectrophotometers are enhanced with an additional monochromator, which controls the wavelengths of fluorescence emitted by the specimen, before it encounters

the PMT. This allows the study of a fluorophore's excitation and emission spectrum. The excitation spectrum describes the efficiency in which light at a specific wavelength excites a fluorophore[165,166]. In concert, the excitation and emission spectra describe peak wavelengths for the optimal detection of fluorophores[165,166].

1.7 STRUCTURE SENSITIVE LUMINESCENCE CONJUGATED OLIGOTHIOPHENES

Luminescent conjugated oligothiophenes (LCOs) are a group of molecular fluorophores developed in the study of disease associated protein aggregates (DAPA)[171-175]. DAPA binding by LCOs occurs via non-covalent electrostatic and hydrophobic interactions and results in a distortion of the flexible thiophene backbone. This generates target-unique optical signatures, as well as an ON-switching of emitted fluorescence[171-173,175]. This binding-dependent fluorescence reporting system has allowed for extraordinary accuracy for both the detection of mature amyloid deposits, as well as the delineation of amyloid of various densities within clinical samples[171,175]. Importantly, LCOs have allowed for the detection of pre-fibrillar forms of amyloid proteins[174,176]. In a disease where clear diagnosis only occurs during post-mortem histological analysis, this unprecedented sensitivity may allow for early detection and medical intervention when the disease is still treatable.

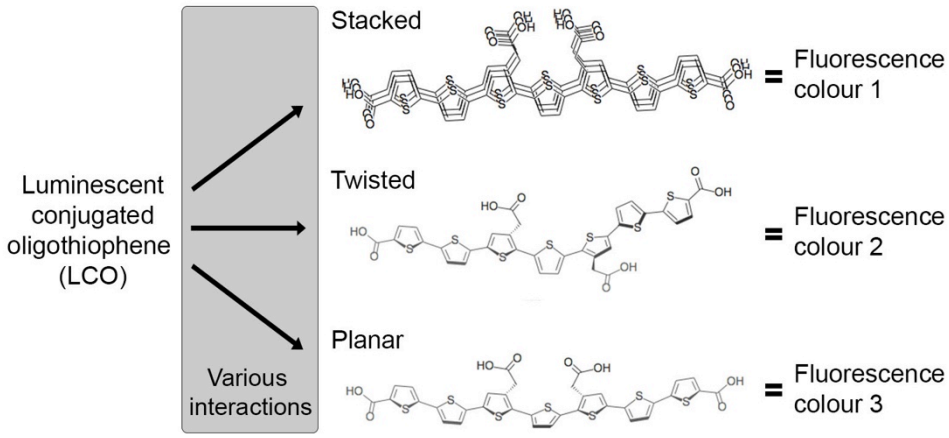

Figure 1.6: The mechanics of luminescent conjugated oligothiophenes (LCO). *LCOs behave as multiple fluorophores based on the twists of the oligothiophene backbone as well aggregative characteristics[172,175]. In a stacked arrangement a typical LCO experiences a red shift in the maxima of the emission spectrum as well as a reduction in emitted fluorescence intensity[172,175]. When adopting a twisted confirmation, an LCO experiences a blue shift in the maxima of the emission spectrum[172,175]. When in a planar confirmation, LCOs experience a red shift in the maxima of the emission spectrum. Often this is associated with an increase in emitted fluorescence intensity[172,175].*

Due to the target dependent distortion in an LCO's molecular geometry when bound, a single LCO can fluoresce at several wavelengths in the visible spectrum[172]. As such, when a single molecular species of LCO binds to several targets, several corresponding fluorescent colors are emitted, wherein each color allows the identification of a specific LCO-target pairing.

LCOs are small and chemically versatile. The commercially available palette of LCOs is represented by mono-dispersed preparations of oligomers of 5 or 7 thiophene units. Selectivity for specific targets can be tweaked by the addition of various side chain functionalities[174,177]. Optical properties of these molecular fluorophores can also be tuned by substitution of thiophenes backbone with heterocyclic motifs[178]. This contrasts against earlier luminescent conjugated polythiophenes (LCPs) that were poly-dispersed and possessed with poorly defined lengths and positioning of functional groups.

2 OBJECTIVES

This thesis is based on 4 papers. Paper 1 covers a little known facet of host-pathogen interactions, studied under the framework of Tissue Microbiological. Paper 2 describes the parallel development of tools and techniques for intravital experimentation. Finally, Papers 3 and 4 shows the trans-disciplinary application of medical research tools to industrial applications.

The Infection Biology aims of this research:

- To establish a dynamic picture of bacterial colonization by identifying the molecular details that orchestrate the changes in tissue homeostasis during bacterial infections
- To visualise biofilm formation in the urinary tract.
- To define the role of selected bacterial virulence factors *in vivo*

The Biotechnological aims of this research:

- To validate the efficacy of luminescent conjugated oligothiophenes (LCOs) for real-time detection of biofilms as they are formed in both *in vivo* and *in vitro* contexts.
- To establish the use of LCOs in the detection of microbial related carbohydrates in both microbiological and biochemical contexts.
- To redirect tools developed for medical reasearch to wider fields of industrial application. Specifically to non-destructive material analysis methods for use in examining materials relavent to to the Cellulose Biorefinery with special focus on lignocellulose biomass.

3 RESULTS AND DISCUSSION

3.1 PAPER 1 – THE NERVOUS SYSTEM: A HIGH-SPEED NETWORK FOR INFECTION COMMUNICATION AND RESPONSE

Paper 1 follows up on the rapid inter-organ line of communication from kidney to spleen described in previous work by Boekel *et al.,* 2011, as part of the host response at early hours of infection[13]. At the site of infection, a delicate balance of molecular pathways governing host responses is brought into play, aimed at resolving the infection, preserving tissue function and minimizing non-specific tissue damage[8,13-15]. Observing changes in splenic transcriptomics, Boekel *et al*., 2011, showed that host responses were not limited to the infection site[13]. Among which there was an increase in systemic levels of IFN-γ, and a distinct up-regulation of IFN-γ and related ILs in splenic tissue biopsies from rats harboring a UPEC infection in a single nephron[13]. However, how this is induced was not described. In Paper 1, we hypothesize that the nervous system was the pathway of communication from kidney to spleen, due to how rapid the change in the spleen's transcriptomic profile (circa. 3 h) was observed. Here the intravital model of infection as developed by Månsson *et al.,* 2007 was instrumental in probing for sensory afferents in the kidney, and testing our hypothesis on a nervous based inter-organ communication and coordination of host responses during infection[8].

3.1.1 Players of Interest During UPEC Infection

To view the infection and guide the selection, designing and testing of primary and immortalized cell culture models, we applied the intravital model of infection as developed by Månsson *et al.,* 2007 together with confocal *ex vivo* imaging[8].

UPEC intruding at the nephron is subjected to strong pressure from the innate immunity and the early-induced innate response[8,15]. Hydrodynamic pressure from glomerular filtration expels majority of bacteria micro-infused into the luminal space. With attachment and proliferation of bacteria at the proximal tubule, infected epithelium are shed and cleared together with renal filtrate and cleared into the distal reaches of the urinary tract. While removing the vast majority of colonizing bacteria, this exposes the underlying basement membrane. Rich in the collagen IV, the basement membrane appeared to contain the infection to the tubular lumen and prevent their dissemination. Activation of the coagulation cascade and stoppage of circulation was observed, similar to what Melican *et al.,* 2008 reported[14]. In parallel, a large number of CD18 positive leukocytes are recruited by signaling

elements of the early immune response to the peri-tubular vasculature. Some CD18 positive leukocytes could also be observed among bacteria in the lumen of the infected tubule.

Looking into long-range communication possibilities in the kidney, confocal *ex vivo* imaging of neural filaments revealed the presence of axonal bodies containing β3-tubulin in the basement membrane surrounding renal tubules, within the juxtaglomerular apparatus, as well as within walls of larger peri-tubular blood vessels. Among the extensive neural infrastructure, we found a subpopulation of sensory neurons. TRPV-1, a marker of sensory neurons was found in close proximity with β3-tubulin. Evidence of sensory afferents in the kidney supported our hypothesis of the involvement of the nervous system for rapid infection sensing and inter-organ communication.

In summary, we shortlisted the following cells and factors as possible players involved in initiating neural communication. These were:

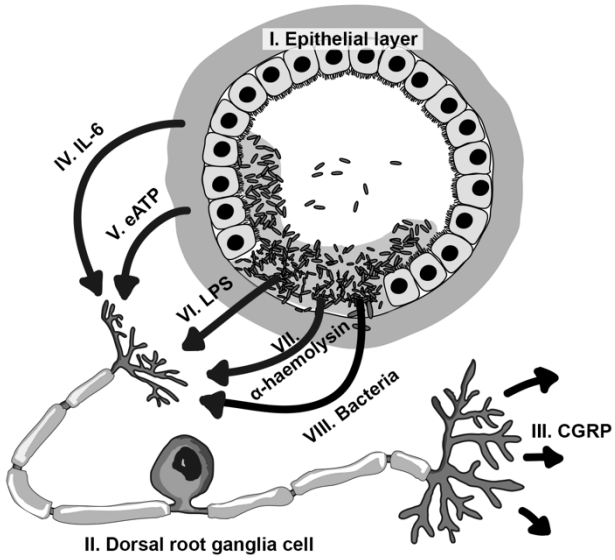

Figure 3.1: Cartoon schematic of the primary infection site. *Sensory projections located in the basement membrane of the PCT may receive a variety of signal from the infected epithelial. We speculate that this may include IL-6, eATP, LPS,* α-haemolysin*, and bacteria. Nerves may then release CGRP as the next line of communication.*

I. Epithelial cells - Transiently present from initial bacteria attachment till they are shed. This was modeled using the immortalized renal epithelial cell line A-498.

II. Sensory nerves - Located in the basement membrane. These were represented by primary dorsal root ganglion cells cultures isolated from BALB/c and C57B/6 mice.

III. CGRP - A neurotransmitter common to nociception.

IV. IL-6 - A known mediator of inflammation and sensitizer of nociception. A role in neuronal differentiation has also been described in existing literature[179,180].

V. eATP – A damage associated molecular pattern (DAMP) and known mediator of inflammation[46,73,181].

VI LPS - A strong activator of the immune response with reported roles as a sensitizer of nociception[92,182-184].

VII. *E. coli* α-haemolysin - A virulence associated factor that induces calcium signalling and cell lysis[185].

VIII. Bacteria – Isogenic strains of the UPEC CFT073 were applied.

3.1.2 Epithelial Initiated Immune Nervous Communication

Infection of epithelia cells (A-498) by the UPEC strain CFT073 resulted in an increase in eATP within 4 h. The eATP originated from epithelial cells and accumulated in the extracellular environment as the infection progressed. By blocking bacterial protein synthesis with tetracycline, we found that eATP release was associated with actively produced bacteria proteins. Further testing with isogenic mutants of CFT073 revealed this protein to be the *E. coli* toxin α-haemolysin. Investigating eATP for neuro-stimulatory effects, we observed significant release of the neurotransmitter CGRP when DRG cells were stimulated with eATP. Our observations linking eATP to neuro-stimulation suggested that UPEC infection at the epithelium may be communicated to sensory afferents of the PNS through an *E. coli* α-haemolysin induced eATP release.

α-Haemolysin is a 107 kDA protein that inserts into mammalian cells, assembling to create 2 nm wide pores[185-188]. While effects are known to vary according to the bacterial species, the general consensus is that α-haemolysin induces cell lysis through swelling by increasing cell membrane permeability. *E. coli* α-haemolysin appears to have an intimate relationship with eATP[185-188]. Skals M. *et al*., 2009 showed that α-haemolysin activates P2X7, a ligand gated cation channel[186]. In Its normally physiological state, P2X7 channels the depolarization of the cell upon activation by ATP[186]. Skals M. *et al*., 2009 concludes that α-haemolysin induces P2 receptor activation, sensitizing mammalian cells to eATP, converting this signaling molecule into a pro-hemolytic agent[186].

In our model, *E. coli* α-haemolysin appeared to have a role in immune evasion. CFT073 infected A-498 secreted visibly lower IL-6 than uninfected controls at 4 h. Further analysis by Live/Dead staining revealed this decrease in IL-6 to be directly proportional to a drop in cell viability. This was in contrast to cultures infected by CFT073 lacking the *hlyA* gene or when active protein synthesis was inhibited, wherein a significant increase in IL-6 secreting was detected. This mechanism of immune evasion by which pro-inflammatory signaling is abrogated by α-haemolysin induced cell lysis corroborated with observation by Melican *et.*

al., 2008. In that study, UPEC lacking α-haemolysin was found to elicit lower inflammation in host tissue than wild type strains[14].

3.1.3 Direct Infection Sensing by Nerves

As the integrity of the epithelium decreases and infected cells are shed, we speculated that LPS might move towards the basement membrane and interact with sensory projections. Stimulation of DRG cells with rough and smooth LPS variants did not induce any change in CGRP release. Instead, an increased secretion of IL-6 was observed. This implied that LPS by itself does not activate neural signaling. This finding corroborated with studies by Rudick *et al*., 2012 and Chiu *et al*., 2014, where LPS was shown to be an agent that sensitizes sensory neurons through the binding to TLR-4[182,183].

As the infection progresses, epithelial cells are shed and bacteria come into direct contact with the basement membrane and the enclosed sensory projections. In our model, DRG cells were infected with CFT073 in the presence of tetracycline to limit the wide range of possible interactions to exposed elements of the bacterial other membrane. In this instance, we observed a significant release of CGRP into the supernatant. Interestingly, DRG cells also had a strong capability for inflammatory signaling. Stimulation with bacteria resulted in an increase in levels of secreted IL-6 at 4 h. Confocal immunofluorescence analysis revealed pre-synthesized pockets of IL-6 in both cell bodies and axon terminals, compartmentalized in the cytoplasm and cytoplasmic vesicles. Importantly, the presence of IL-6 at the axon terminals suggested the possibility for quick release following stimulation. This related to published findings of Chiu *et al*., 2014, showing anti-inflammatory effects of CGRP fed back from neurons to the primary infection site[182],

Similar to Chiu *et al*., 2014's work, preliminary findings from a protein array analysis we performed (data not shown) showed the release of the immune-modulatory cytokines CXCL10, CXCL1, CXCL2, MCP-1 and TIMP-1. Confocal immunofluorescence analysis showed that MCP-1 and CXCL2 was pre-synthesized and compartmentalized in the same way as IL-6. This protein secretion pattern was unique to DRGs and was not observed in A-498 cells. Our findings here implied that sensory afferents had both neural and immune roles. DRGs could both sense and neurologically communicate surface exposed bacterial factors and contribute to the early-induced innate response by reporting homeostatic changes in the tissue via a range of cytokines.

3.1.4 A Neural Connection from Kidney to Spleen

In Paper 1, we hypothesized a communication pathway wherein an infection at the epithelium induces a first wave of signaling factors that stimulates sensory neurons in the basement membrane that in turn activates a neural based communication from kidney to spleen. This was based on our finding of an *E. coli* α-haemolysin induced secretion of eATP by epithelial at 4 h that is likely to activate neural communication and subsequent CGRP release. Checking for splenic IFN-γ mRNA as the indicator of kidney to spleen communication, we found strong evidence that the infection in the kidney was communicated to the spleen. qPCR analysis of biopsies at 4 h showed that infusion of CFT073 into a single nephron at the kidney resulted in a significant 3 fold increase in IFN-γ mRNA transcripts at the spleen. No change in splenic IFN-γ transcription was observed when an isogenic mutant lacking the *hlyA* gene was infused into the nephron. We concluded that kidney to spleen communication was dependent on intact α-haemolysin expression. Combining this finding with the detected patterns of epithelial eATP release described in earlier sections of this Paper, we saw strong evidence for a neural pathway communicating infections at kidney to the spleen.

3.1.5 Conclusion

The communication between the immune and nervous systems is a rapidly emerging field that is gaining increased recognition as an important player in the immune response[77]. Traditionally, signals of the early-induced innate response are thought to circulate hemodynamically to 'effector' organs, at which a cascade of anti-infection responses is put into motion[62]. Said responses then re-enter circulation, to exert its effects at the primary infection site. Paper 1 demonstrated the role of the sensory neurons in the periphery for detecting infections and communicating this to distal organs via the CNS. UPEC colonization in the kidney can occur extensively in the renal cortex and medulla, throughout the nephrons' labyrinth-like nature. At any one site, a step-wise series of events ensue, the severity of which may vary greatly. We developed 4 h models of infection *in vitro*, which mimics the *in vivo* phases of innate immunity and early-induced innate response that can occur at 0–4 h and 4–96 h respectively[46]. Analysis of the inflammatory responses of key cell types at the infection foci and the subsequent comparison revealed individual differences of each cell's role in detecting and responding to the infection. In doing so, we were also able to observe an *E. coli* α-haemolysin dependent electrical activation of DRGs through epithelia secreted eATP that we think communicates the presence of pathogenic *E. coli* strains to the CNS. This information is then relayed to distal organs wherein the appropriable host responses are

initiated. Looking back at Boekel *et al*., 2011's, the infection at the kidney induces the production of IFN-γ at the spleen[13]. This resulted in an increase of IFN-γ in circulation that is linked to the activation of IFN-γ related genes at infected kidney[13]. IFN-γ coordinates a wide range of infection response programs such as up-regulation of pathogen recognition, antigen recognition and leukocyte trafficking[59]. Paper 1 thus contributes to the understanding the pathophysiology of pyelonephritis during time points prior to the reciprocal influx of systemic immune elements into the primary site of infection, a window that is to date still not well understood.

3.2 PAPER 2 – BACTERIAL POPULATION BEHAVIOR IN INFECTION: DEVELOPMENT OF TOOLS TO STUDY THEM

Paper 2 focused on the life style of bacteria *in vivo,* and was conducted in parallel to Paper 1. Intravital imaging of UPEC colonization of the hydrodynamically challenging renal tubulosystem by Månsson *et al.,* 2007, as well as subsequent applications of the model by Melican *et al*., 2011 emphasized the multicellular quality of bacteria within the *in vivo* systems and the role of biofilms in surviving physically challenging microenvironments[8,15]. The term biofilm is loosely defined as an organized unit of bacterial cells and its extracellular matrix (ECM)[125-127,133]. To be able to conduct an intravital study of biofilm population behavior during an infection, we first had to develop a tool that would allow for real-time tracking of the biofilm and its components. This is because common techniques currently used to detect biofilms use general dyes that do not diffentiate bacteria from their ECM, or the multicellular biofilm from the planktonic lifestyles. These dyes are also often not suitable for *in vivo* use.

Paper 2 describes the establishment and verification of a method to detect the biofilm ECM, with the goal of eventual use in intravital and *in vivo* infection studies. In addition to the detection of biofilm ECM components as a prerequisite, we set 3 criteria to be met.

I. Non-disruptive use in biofilm cultures
II. A distinct readout that is easy to identify.
III. Scalable for high throughput screening and continuous tracking.

To develop the Optotracing methodology, we used *Salmonella enteritidis (S. enteritidis)* strain 3934, a well-established model that expresses a curli and cellulose rich ECM. The oligothiophenes h-FTAA and h-HTAA were selected as potential candidates for biofim detection. Both molecules belong to a group of conformationally sensitive oligomers known as luminescent conjugated oligothiophenes (LCOs), traditionally used to study miss-folded amyloid proteins in Alzheimer's disease[172,174-176,178,189]. Biofilms were grown on a glass coverslip by an 'inclined coverslip' setup to allow subsequent analysis of biofilms at the air-liquid interface by microscopy. In this preliminary phase of testing, fixed biofilms were stained simply by 30 min incubation in PBS containing h-FTAA or h-HTAA. This resulted in distinct fluorescence reporting of the gross biofilm morphology when viewed under a confocal microscope. Comparing LCO stained biofilms of isogenic strains of *S. enteritidis* 3934, we observed morphological differences between various biofilms that corresponded to the expression of different ECM components. Furthermore, the signal intensity of both h-HTAA and h-FTAA in the biofilm was directly correlated with the strains ability to produce

cellulose (*ΔcsgA*) and curli (*ΔbcsA*)[190-192]. Indeed, no fluorescence was detected in the biofilm of *S. enteritidis* 3934 *ΔcsgD*, wherein both cellulose and curli were absent due to a delection in *csgD*, the biofilm master regulator[190-193].

3.2.1 LCOs are neither Antimicrobial nor Inhibitory to Biofilms

For use *in vivo*, we required LCOs to be non-disruptive towards bacteria lifestyles. Tracking the growth rate of *S. enteritidis* 3934 cultures in the presence of h-HTAA or h-FTAA, we found that neither LCOs affected bacterial division time or cell viability. To check if LCO affected biofilm formation, we designed a 96-well assay to allow endpoint quantification of pellicle and surface attached biofilm by recording fluorescence of biofilm bound LCOs emitted at λ_{Em} 556 nm when excited at λ_{Ex} 405 nm. Endpoint quantification of several parallel cultures of *S enteritidis* 3934 biofilm at several time points showed a gradual formation of biofilm beginning at 10 h which peaked at 30 h. This pattern of formation was identical to the trend obtained from the CV assay conducted in parallel as reference. We found that LCOs were comparable to the CV assay in differentiating isogenic mutants of *S enteritidis* 3934 with phenotypic differences in cellulose and curli expression. Here, the LCO method appeared to have added resolution for curli only biofilms. The assay detected significant amounts of biofilms produced by the *ΔbcsA* strain, which was not picked up by CV assay.

Continuing the path to developing live *in vivo* biofilm reporters, LCO signals were read directly from unwashed *S enteritidis* 3934 cultures in the 96-well assay, to identify biofilms produced without physical manipulation of the sample. In doing so, we further narrowed our reporter of choice. Without the removal of the planktonic bacteria, h-HTAA was unable to detect the phenotypic differences of biofilms between *S enteritidis* 3934 and isogenic stains lacking cellulose (*ΔbcsA*), curli (*ΔcsgA*), or both curli and cellulose (*ΔcsgD*) in the ECM. h-FTAA stood out as our reporter of choice, reproducing data from the washed 96-well assay shown earlier. Additionally, h-FTAA detected distinct amounts of biofilm produced by the *ΔcsgA* strain that was again not detected by the CV assay.

Factoring in the extensive research on LCO detection of DAPA, that showed no cytotoxicity in eukaryotic *in vitro* models or *in vivo* mouse models[194]. We concluded that LCOs seemed ideal for live bacterial studies, as well as eventual intravital applications.

3.2.2 LCOs Produce Distinct Optical Signatures When Bound to the ECM

In-depth scrutiny of the excitation spectrum of h-FTAA within each biofilm culture showed that h-FTAA binding to curli and cellulose could be identified at well-separated optical windows by applying defined excitation wavelengths. When bound in biofilms expressing cellulose, h-FTAA exhibited a highly red shifted excitation spectrum, showing an Ex. λmax at 480 nm and an emission spectrum with dual peaks at 560 and 600 nm. In contrast, binding of h-FTAA in biofilms expressing curli results in an increase in signal intensity at λ_{Ex} 405 nm compared to curli deficient strains. While less distinct compared to cellulose, dual peaks in the emission spectrum was also observed at 560 and 580 nm. These patterns in the excitation and emission spectra form the basis of an optical signature, used in the Optotracing method developed in Paper 2 to identify ECM compositions in biofilms.

CR is a long-standing biofilm classification method by which a biofilm's phenotype can be defined by its physical appearance as the CR dye incorporates into the biofilm[154]. Termed Rdar morphotyping, the method is dependent on the analysis of physical features of the biofilm when it reaches sufficient size for visual inspection. When applied to *S. enteritidis* 3934 biofilm colonies grown from a single bacterium, this typically occurs after 48 h. Optotracing matched this sensitivity, the optical signature of h-FTAA when bound to the ECM, clearly indicated the presence of cellulose in biofilms grown for 48 and 72 h. Application of h-FTAA at the 24 h time point was even able to show the presence of cellulose in the minute colonies that were at this time barely distinguishable from the head of a pin. Further analysis of these 24 h biofilm colonies showed a non-uniform distribution of cellulose expression across the population on the plate. A subpopulation of tested colonies showed intermediate degrees of red shifts in the h-FTAA excitation spectra. Optotracing for biofilm cellulose showed that phenotypical difference in ECM can still occur in biofilms grown from single bacterium under the same conditions. While curli detection was not demonstrated due to the high level of background signals, h-FTAA still seemed a more straightforward means than the CR assay.

3.2.3 The LCO Assay Allows Continuous Tracking of Biofilm Formation

Parameters to separate optical signals of different forms of bound h-FTAA were extensively characterized in Paper 2. Fluorescence signals of h-FTAA arising from curli binding could be detected at λ_{Ex} 405 nm and λ_{Em} 556 nm, while signals from cellulose binding could be detected at λ_{Ex} 500 nm and λ_{Em} 600. The use of these parameters enabled us to view in real-time the trend of curli and cellulose production by the growing bacterial (*S. enteritidis* 3934 p2777) culture as it enters stationary phase. The method described in Paper 2

allowed continuous (hourly) non-disruptive readings and was sufficiently sensitive to detect compensatory mechanisms set in motion in the absence of either ECM components. In *S. enteritidis* 3934 lacking curli expression (*ΔcsgA*), biofilm formation occurred significantly earlier during exponential phase, and at a faster rate. Conversely, *S. enteritidis* 3934 lacking cellulose expression (*ΔbcsA*) appeared to produce biofilm at a slow consistent rate that appeared disconnected/independent from the common sigmoidal pattern of bacterial growth. This level of resolution showing the kinetics of ECM formation was first of its kind.

3.2.4 Applying LCOs to Detect Intracellular Cellulose

Moving closer to our goal of intravital testing, h-FTAA was adapted for use in *in vitro* culture settings. We adopted *S.typhimurium* 14028[195,196], a facultative intracellular pathogen to track movements of this bacteria's behavior during infection. h-FTAA profiling of *S. typhimurium* 14028 revealed the presence of curli and cellulose within the biofilm, which we tracked in real-time and visualize by CLSM. We hypothesized that h-FTAA may be sufficiently sensitive to cellulose present in during a pathogens' lifestyle *in situ*. By means of h-FTAA tracking, intracellular *S.typhimurium* 14028 could be detected in renal epithelial cells (CRL4031), hepatic macrophages (RAW 264.7) and hepatic tissue (mice). As a result, h-FTAA revealed the expression of cellulose within Salmonella containing vacuoles (SCV).

3.2.5 Conclusion

Expression of ECM is a key step in bacteria's transition from planktonic to a sessile biofilm lifestyle[130,133]. When assembled, the ECM becomes functional component of the biofilm thought to protect enclosed bacteria from antimicrobials, chemical agents and mechanical removal strategies[125-127,133,141]. Despite the annually growing number of studies, few methods exist that can specifically study this biofilm component[148]. Most methods also depend on the physical separation of planktonic phase of the culture from the sessile biofilm before analysis[148]. The sample destructive nature of this step disallows continuous experimentation. Paper 2 describes a game-changing technology in the study of biofilms. The chameleon like feature of LCOs produces a unique optical signature identifying two critical components of the ECM, without the need for physical manipulation to aid resolution. The described Optotracing technology showed the dynamic and kinetics feature of ECM formation as the biofilm develops, and compensatory changes in biofilm phenotype when expression profiles are changed by genetic manipulation. In a field wherein mainstay methods depend on visual analysis by experienced diagnosticians and the use of cytotoxic/carcinogenic dyes, Optotracing is set to be a safer, more accessible method with lower user bias.

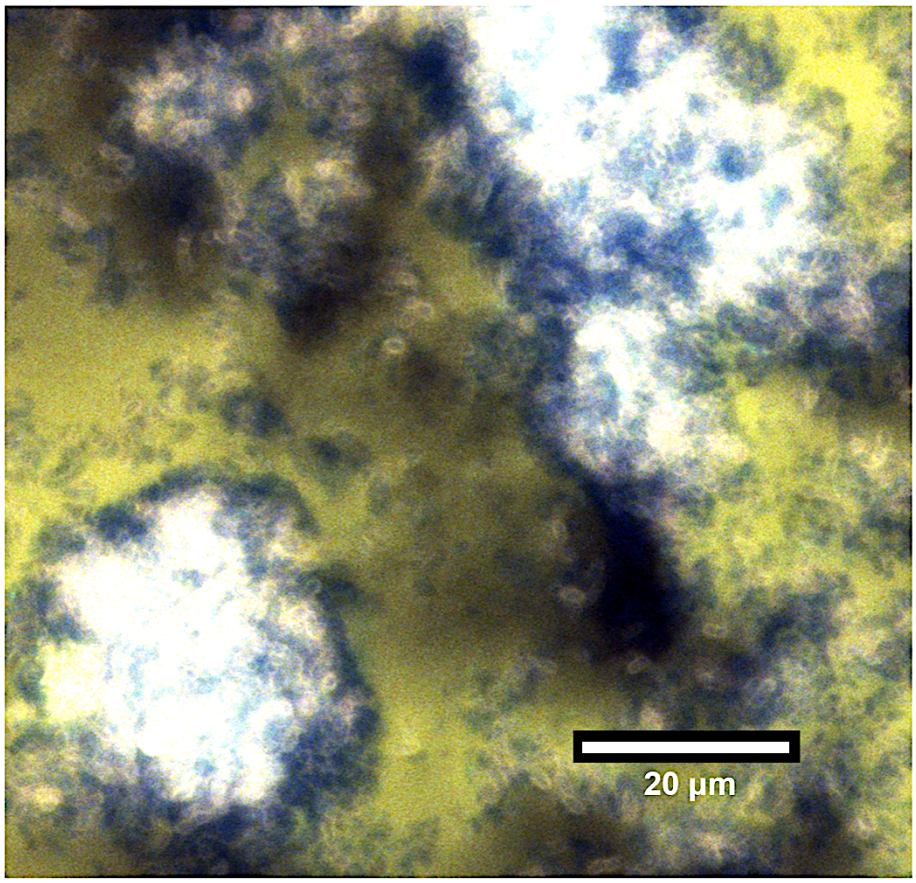

Figure 3.2: S. enteritidis 3934 p2777 biofilm stained by h-FTAA at 48 h showing cellulose (yellow) and curli (blue). *Grown in LB without salt at with the 'inclined coverslip setup' and imaged on a fluorescence microscope equipped with a SpectraCube module. Excitation is applied at 405 nm and emission is collected between 415-750 nm. Pseudo-colors were applied automatically by the software according to the signal intensity and emission wavelengths.*

3.3 PAPER 3 – TRANSCENDENT OPTOMETRIC TOOLS FROM BIOFILMS TO FORESTRY

Paper 3 follows and expands on findings reported in Paper 2. Optotracing with LCO, was a sensitive method for detecting bacterial cellulose. In a curiosity and innovation driven project, Paper 3 describes reapplication of tools developed for medical research for a wider range of uses. Through web searches, scientific literature reviews and interviews, we identified the Cellulosic Bio-refinery as a field wherein Optotracing could find use as an *Enabling Technology*[197-200]. We endeavored to transfer 3 features common in tools for medical research; these were (I) high optical resolution, (II) low volume, and (III) the possibility for high throughput.

The Cellulose Bio-refinery concept aims to increase manufacture of ethanol from cellulose and non-cellulose carbohydrates, optimize processes, and to maximize the number of uses that can be acquired from a biomass[197,200-203]. The push for the Cellulose Bio-refinery concept comes as World economies acknowledge the finite future of fossil fuels[204,205]. Plants and plant biomass that are rich in polysaccharides are a source of biofuels and 'bio-plastics', and are viewed as a potential fossil fuel alternatives[200,204-211]. In order to maximize the full range of plant based materials that can be obtained, extensive research is needed to optimize extraction and synthesis processes, and profile the qualities of materials produced. Unfortunately, this is difficult with technology and methods available today[212-215]. In a recent review of modern biomass fractionalization and valorizations, authors pointed out the critical need of the research field to mature, to which the lack of tools is one major obstacle[197].

3.3.1 The Cellulose specific reporter p-HTEA

In Paper 3, we focused on cellulose detection across the forestry, pulp and paper, and the biofuels industries[200,216]. Cellulose is among the most abundant materials in nature and is of great economic importance. We selected p-HTEA, a cationic pentameric LCO, to develop the Optotracing method for cellulose detection. When bound to microcrystalline cellulose (M. cellulose), p-HTEA exhibited a distinct and unique optical signature. This was composed of a red shift in Ex. λmax, and an increase in signal intensity that was directly proportional to the amount of M. cellulose tested.

Interestingly, the optical signature of p-HTEA was identical across the various cellulose morphologies tested, be it M. cellulose, pulp cellulose or nanocellulose. This was also shown during CLSM imaging of p-HTEA, wherein M. cellulose, pulp cellulose fibers, nanocellulose and nanocellulose paper, emitted identical fluorescence qualities when excited by a laser at

473 nm. Signals from bound p-HTEA in each sample was intense, and allowed detailed visualization of surface features of each cellulose material. This gave us reason to believe that p-HTEA binds directly to the individual chains of β(1→4) linked D-glucose polysaccharide.

3.3.2 Detection of Lignocellulose Biomass by p-HTEA

Observations of Paper 2 suggested that p-HTEA would be able to selectively detect cellulose in nature. Cellulose in plant cells exists as a heterogeneous matrix, in which cellulose fibers alongside hemicelluloses are cross-linked by lignin[217-219]. This state is commonly referred to as lignocellulose biomass. Lignin is a complex, polydispersed and heterogeneous polymer composed principally of phenolic compounds[218]. During lignocellulose biomass fractionation, strong cellulose fibers of varying degrees of purity are designated as the main revenue streams, while lignin and hemi-cellulose rich run-offs are considered low value streams[197,216]. We observed distinct auto-fluorescence when analyzing unstained samples of lignin. This was likely due to the abundance of phenol motifs in lignin[218]. We found that Kraft lignin absorbs maximally at 339 nm and emits fluorescence at 545 nm, of which the signal intensity was directly proportional to the concentration present. Unlike cellulose, lignin binding quenches p-HTEA. We observed a concentration dependent quenching p-HTEA, to the point where complete loss of unbound p-HTEA optical signature was achieved by 0.15 mg/ml of lignin. Addition of lignin to p-HTEA pre-bound cellulose competitively 'stripped away' p-HTEA and eliminated the cellulose specific optical signature. This implied that the binding affinity of p-HTEA to lignin was higher than that of cellulose. Similar to lignin, hemicellulose was found to posses an affinity for binding p-HTEA, quenching its intrinsic fluorescence. Hemicelluloses are polysaccharides build up of xylose, mannose, galactose, rhamnose, arabinose and glucose[216]. Of 3 hemi-cellulose types tested, galactomannan and xylan was found to possess higher affinity of cellulose binding than O-acetylgalactoglucomannan.

p-HTEA appeared highly sensitive to the presence of non-cellulose polysaccharides in plant material. Results of Optotracing and competitive binding assays implied that cellulose specific optical signatures of p-HTEA would only appear in fractions of lignocellulose biomass wherein cellulose is the principle component, and lignin and hemicelluloses have been removed sufficiently. This was reminiscent of pulping process, during which lignin and hemicelluloses are separated from cellulose in a digester and channeled away as liquor A.K.A. low value streams[197,216,220]. Pulp cellulose extracted in this process is then bleached and dried to remove residual lignin and water[216,221]. Mimicking certain aspects of the process, we applied Optotracing to several fractions obtained from a local pulp and paper mill for the

presence of cellulose. Doing so, we found no indication of cellulose in liquor samples that contained lignin hemicellulose as the main constituents. Surprisingly p-HTEA did not produce the optical signature in unbleached pulp despite the high cellulose content. This confirmed that p-HTEA is highly sensitive to the purity of cellulose in the sample, such that a low 4 % presence of lignin (Kappa: 27.9)[222,223] was not tolerated. The optical signature of cellulose was only observed after further de-lignification had been performed, shown in analyzed samples of bleached pulp (Kappa<2)[222,223]

3.3.3 Continuous Tracking of Cellulolysis by p-HTEA

The conversion of cellulose to glucose is a key step in the production of biofuels[208,210]. This motivates a wide variety of research into chemical processes and enzymes to identify low cost methods and ways to maximize yield[210,224,225]. Paper 2 showed that LCOs could monitor changes in biological systems in real time. This meant that p-HTEA could be added into mixtures of active enzyme and cellulose to monitor the efficiency and kinetics of the reactions. In a quick and easy set up, we demonstrated optical tracking of the enzymatic digestion of M. cellulose, nanocellulose, and nanocellulose paper by 1–8 units of cellulase originating from *Trichoderma viride*. Intense cellulose specific fluorescence detected at the initial point of the experiment decreased distinctly over time in proportion to the enzyme's amount and rate of reaction. This implies that p-HTEA binding was reversible, and did not affect the enzyme's function. Since LCOs reporting did not interfere with cellulolysis, LCOs seemed like an ideal tool in the development of better cellulases.

3.3.4 Conclusion

A main theme in Paper 3 was the transference of inter-disciplinary technology from Medicine to the Cellulose Bio-refinery. We were able to show utility of Optotracing with LCOs for material analysis in several lignocellulose processes at the entry-level. The focus of this study was not to answer in-depth questions on the biochemical phenomena of cellulose sensing by LCO but to produce data that would become the foundation for future studies and the improvement of the current state of art.

With renewed interest towards lignocellulose biomass as a renewable alternative to fossil based resources, we as well as other groups foresee an impending need for better tools for researching of this material[197-199]. Studies to optimize the use of lignocellulose biomass as materials or as fuels will require real-time methods to directly measure the consumption of cellulose. This is not trivial, as it is difficult to distinguish different polysaccharides composed of glucose using current methods[212-215,226-228]. Methods often require tough sample

preparation and requires sizable amounts of sample for analysis[214,215]. Similarly, there is also a need for better tools in the pulp extraction pipeline[197]. In this industry, phase contrast based imaging to estimate the fiber dimensions, alongside Kappa number determination, are the mainstay methods for in-line tracking of proper operations[216,220,221]. When necessary, samples are extracted and sent to specialized in house or external laboratories for more accurate evaluation by ion exchange chromatography or gas chromatography-mass spectrometry[214,215]. This time lag between detecting errors and error resolution results in significant wastage when batches have to be recalled.

Applying approaches in medical research, we were able to achieve our aims of transferring a number of advantages and show a range of scenarios where Optotracing is useful in the Cellulose Bio-refinery field. Optotracing was surprisingly versatile in small volume systems. Methods described in Paper 3 demonstrated the possibility of direct material analysis, with minimized sample consumption and high throughput in the research setting. These advantages are equally applicable to the manufacturing processes, where there is a need for real time, in-line monitoring of processes to be able to ensure the consistent and optimal functioning. We foresee that Optotracing with LCOs will confer the opportunity to reduce waste by shortening the time between detecting errors and resolving them. It will be an interesting next step to verify if LCOs can be added alongside feedstocks during pulp extracting. Optotracing may be useful in the large volume systems used in pulp extracting, to track changes in cellulose composition as each value stream is produced.

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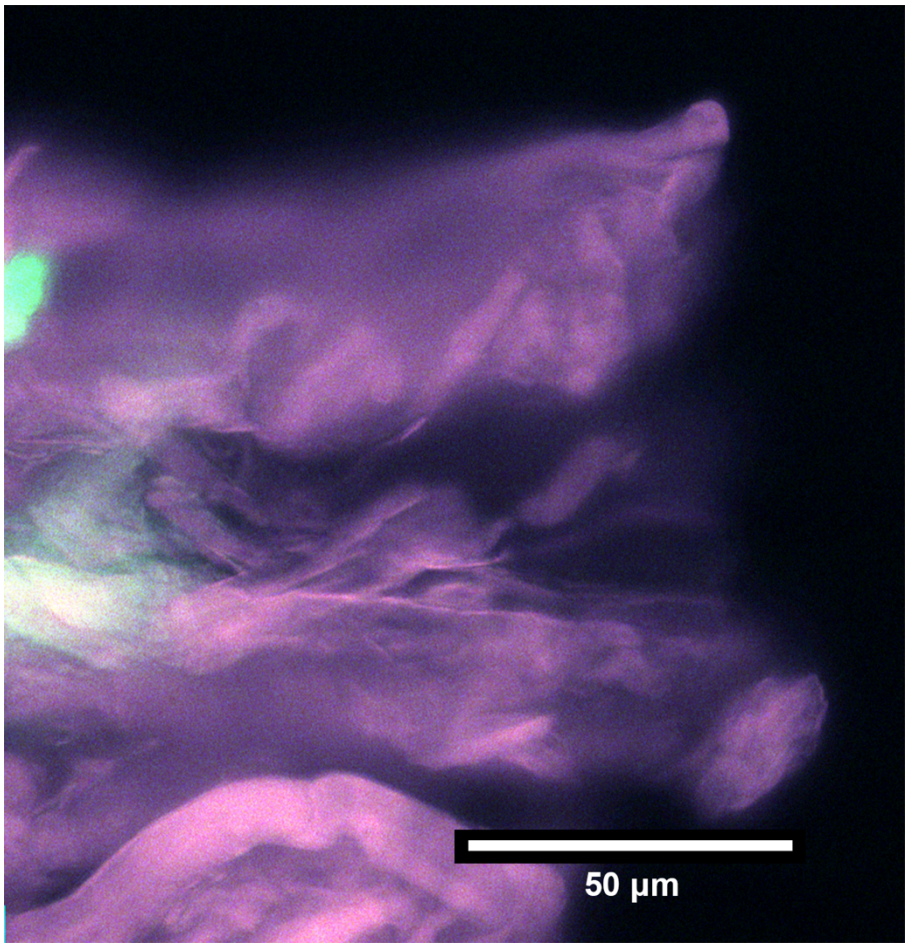

Figure 3.3: M. Cellulose stained by an LCO (purple) *Imaged on a fluorescence microscope equipped with a SpectraCube module. Excitation is applied at 436 nm and emission is collected between 451-800 nm. Pseudo-colors were applied automatically by the software according to the signal intensity and emission wavelengths.*

3.4 PAPER 4 – OPTICAL DETECTION OF MOLECULAR DIFFERENCES IN POLYSACCHARIDES

Paper 4 explores the molecular details of LCO interactions that allow cellulose Optotracing that was previously developed and applied in Papers 2 and 3. There, Optotracing with LCOs was shown as a novel methodology for the non-destructive analysis of material composition. A binding induced change in molecular geometry of LCOs when bound to cellulose provided unique an optical signature that allow accurate detection of cellulose. We exploited this unique reporting mechanics in Paper 4 for further development of Optotracing methods for material analysis. We termed the resultant series of methods as 'Carbohydrate Anatomical Mapping'

Cellulose binding by h-FTAA results in an optical signature with several distinct features. When read in a spectrophotometer, we observe:

I. an OFF/ON like increase in signal intensity.
II. red shifts in both excitation and emission spectra from the optical signature of the h-FTAA unbound state
III. an Ex. λmax at 497.5 nm flanked by shoulders at 460 and 525 nm, and a dual Em. λmax at 558 nm and 593 nm.

The spectrophotometer, which has relatively low microscopic resolution, detects the optical signals of all bound and unbound LCOs in a sample. We hypothesized that in this setup, the observed optical signature of cellulose was the sum of all sub-populations of LCO geometries that may be present, including the unbound form.

To test this hypothesis, we used a fluorescence microscope equipped with a SpectraCube module to perform Spectral microscopy. This method visualizes samples by collecting the emission spectrum of all fluorophores in a sample that fluoresces when excited by a specific wavelength. The spectral microscope system then applies pseudo-colors to the fluorescence micrograph based on the signal intensity and emission wavelengths of each fluorophore. Under Spectral microscopy, bound and unbound forms of LCOs can be differentiated by colour, based on the unique optical signature of each geometry. Spectral microscopy of h-FTAA stained crystals of M. cellulose showed the presence of 2 binding sites, indicated by 2 distinctly colored regions. This implied that h-FTAA bound at the crystal edge had distinctly different geometries from h-FTAA at the crystal core.

3.4.1 Multi-Laser/Detector Analysis of h-FTAA Geometries

Optotracing of different bound forms of LCOs by Spectral microscopy is exceedingly easy. Yet as the equipment and software is expensive and not widely available, uptake of the method is likely to be slow and limited. CLSM setups in comparison are much more common and affordable. In Paper 4 we undertook the development of a CLSM based method to be used as a more accessible alternative to Spectral microscopy. Termed 'Multi-laser/detector analysis', the method visualizes various bound geometries of h-FTAA using several independent excitation wavelengths. In this way, cellulose bound h-FTAA fluoresces strongly when excited by 473 nm and 535 nm lasers. Differences in the location of fluorescence collected in 473 nm and 535 nm on the M. cellulose crystal suggested the presence of at least 2 distinct binding sites. Multi-laser/detector analysis produced images similar to those from the spectral microscope, albeit weaker resolution of colors and poorer discrimination of h-FTAA bound at the crystal edge and the core.

3.4.2 h-FTAA Interacts Directly with the Polysaccharide Backbone of Cellulose

A goal of paper 4 was to determine the binding site and binding ratio between LCO and cellulose. This information was essential to correlate optical signatures and signal intensities with the mass of cellulose in complex mixtures, as they exist in nature[167,218]. Here we studied the optical signatures of h-FTAA when in interaction with several cellodextrans, which are short lengths of oligosaccharides produced during the enzymatic degradation of cellulose. The optical signature of h-FTAA bound to M. cellulose was used as a positive standard for comparison. Comparing the optical signature of h-FTAA when bound to cellopentaose, celloheptaose and cellootaose, we determined that a minimum of 8 glucose units were required to reproduce the optical signature of M. Cellulose. Cellodextrans of 7 units and below induced intermediate red shifts in Ex. λmax.

Applying carboxymethyl cellulose of different degrees of substitution, we determined that accessibility of h-FTAA to the cellulose backbone was essential for the cellulose specific optical signature. Extrapolating these findings, we speculated that h-FTAA may be be useful in tracking production of cellulose esters (A.K.A. cellulose plastics). Continuous tracking of reactions would likely show a gradual decrease in cellulose specific h-FTAA signals as cellulose is gradually converted in to the product.

3.4.3 Carbohydrate Anatomical Mapping of Cellulose

h-FTAA was able to both detect the presence of different R-groups on cellulose as well as detecting differences between glucose polysaccharides of α and β configurations, by correlating signal intensity against Ex. λmax produced by h-FTAA in each interaction. Polysaccharides of the β configuration induced dramatic red shifts in Ex. λmax and a distinct ON switching of fluorescence that was not observed with those of the α configurations. This was an interesting advantage over the state of art techniques used to characterize polysaccarides. Techniques such as IC and GC-MS, which predict the identity of the polysaccharide based on the constituent building blocks, are unable to distinguish polysaccharides composed of the same monosaccharide[214,215]. To demonstrate the ability of h-FTAA to distinguish between glucose polysaccharides of α and β configurations in plant tissue, we applied the Optotracing technology to image potato parenchyma and selectively visualize cellulose in this starch rich tissue. Multi-laser/detector analysis showed bright fluorescent cellulose bound h-FTAA within the cell wall. In contrast, large abundant starch granules present the cytoplasm that was seen under phase contrast remained non-fluorescent and were invisible under Multi-laser/detector analysis. Due to its small size and high photostability, h-FTAA was able to show miniscule details on the cell wall. Not only were numerous plasmodesmata visible, ranging from 0.5 to 4 μm in diameter, fine textures of the cellulose cell walls could also be visualized. Herein was proof of concept of a cellulose specific form of Carbohydrate Anatomical Mapping of materials by Optotracing.

3.4.4 Conclusion

Advancing from Paper 2 and 3, Paper 4 provided molecular evidence showing that h-FTAA interacts directly with the polysaccharide backbone of cellulose. Paper 4 showed that h-FTAA binds by aligning to the polysaccharide backbone with a minimum of 8 β(1→4) linked glucose oligosaccharides. This mechanism of binding to cellulose is likely to induce a highly linear h-FTAA geometry that would account for the drastic red shift in Ex. λmax that we have observed throughout Papers 2, 3 and 4. Imperfect alignment can occur and is detected as intermediate shifts in Ex. λmax.

Taking advantage of the small size and high quantum yield of h-FTAA as a fluorescence reporter, we were able to perform high resolution imaging of cellulose compartments in plant tissues[167,229]. This is a considerable improvement over other similar dyes such as Calcfluor and Hoechst 33358 that indiscriminately binds a range of polysaccharides in plant tissues[167,229]. Showing simultaneous visualization of lignin, chlorophyll and cellulose in plant tissue, Carbohydrate Anatomical Mapping with h-FTAA

is a first step towards a simple and visual method to simultaneously quantify several components of biomass. Maximizing the usability of Optotracing was a key goal of Paper 4. As such, we developed several methods for which h-FTAA could be applied for the detection of cellulose within the materials. The methods as described, could be performed using a spectrophotometer, fluorescence microscopy or a CLSM system. We envision that most laboratories would possess one or both equipment.

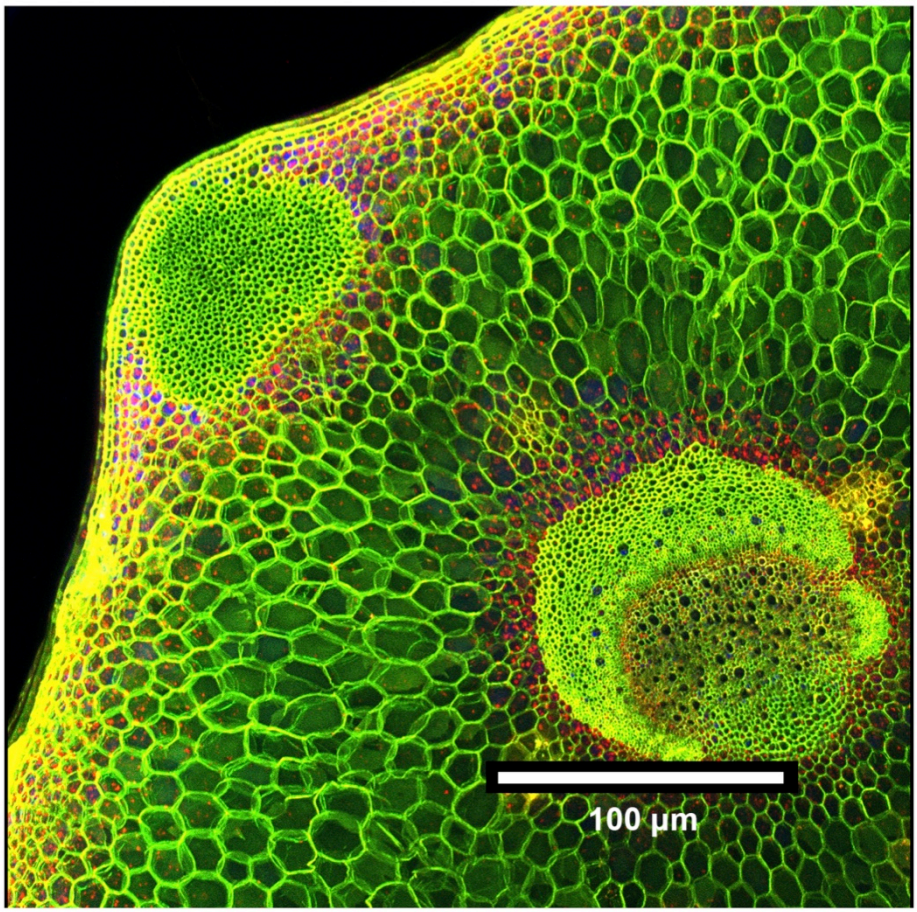

Figure 3.4: Celery stained by h-FTAA. *Celery purchased from a local supermarket cut to 1 mm thick sections and stained for 1 h with 5 µM of h-FTAA in PBS. The stained section is imaged by Multi-laser/detector analysis described in Paper 4. The 405, 473, 559 and 635 nm lasers were applied and pseudo colored blue, green yellow and red respectively. The image stack shows a cellulose specific (yellow green) Carbohydrate Anatomical Map against chlorophyll (red) auto-fluorescent molecules and other pigments in the tissue.*

4 CONCLUSIONS AND PERSPECTIVES

The papers included in this thesis describe two lines of Tissue Microbiology inspired research. In one line, the intravital infection model acted as a guide for experiment design and revealed a role of the nervous system in the kidney that senses and communicates infections to distal organs to initiate a targeted systemic response. In another line, the need to study multicellular bacterial lifestyles *in vivo* motivated the development of novel tools. Further characterization of these tools then led to an inter-disciplinary technology transfer between man, microbe, and plants.

4.1.1 The Nervous System in Infection Response

Studies of the neural pathways in sensing and communicating infections have revealed the importance of is circuitry in regulating systemic inflammatory signals and controlling sepsis[17-24,117,118,121,230]. Surprisingly, this had not been shown at the kidney at the time this thesis was written. One of the first significant findings in this thesis was the co-localization of sensory afferents and the infection site. The primary site where bacteria are micro-infused experiences many changes as the infection progresses[8,13-15]. It appeared that these changes as such are conveyed immediately to the underlying sensory network surrounding the PCT. While we were only able to study a small subset of possible signaling factors, it was apparent that sensory nerves were highly versatile and in sensing a variety of PAMPs, DAMPs, cytokines and chemokines. The involvement of the nervous system in inducing the appropriate systemic responses from distal organs to the specific bacteria phenotype may explain earlier observations by Melican *et al*., 2011, wherein the endpoint of infection clearance of virulent and non virulent stains is identical. Variations in neural signaling pathways tailored to the invading bacteria may also account for asymptomatic states or different symptoms manifested during pyelonephritis. In addition, we were able to show a direct link between the *E. coli* toxin α-haemolysin and the splenic expression of IFN-γ. This contributed to uncovering the inter-organ communication loop described by Boekel *et al* 2011 that connects the kidney and spleen. In the specific case of UTI, this work has brought up several possible lines of investigation for future studies.

- Further characterisation of the types of sensory nerve fibers in the kidney.
- Locating the anatomical pathway of nerves that communicate infections from the kidney.
- What other PAMPs, DAMPs and cytokines activates sensory afferents?
- Do cytokines secreted fom nerves have an effect on the primary infection site?

- What are the effects of splenic IFN-γ in the primary infection site?
- Does the blocking of kidney-spleen communication have a visible effect on the infection dynamics?

4.1.2 Optotracing of Microbe and Plant Materials

The Tissue Microbiology approach is a powerful platform for the study of infections[5-7,231]. The twophoton-based model in particular provides a cinematic view of host-pathogen interactions. Unfortunately the range of *in vivo* fluorescence markers remains few to this date. This motivated the development of the Optotracing technology. In this thesis, we focused on the ECM components of biofilms. Specifically, we were able to selectively visualize curli and cellulose in live biofilms by microscopy and track their formation in relation to bacteria growth phases. This revealed interesting new dynamics of biofilm formation, and addressed some shortcomings of the existing methodology for biofilm studies.

Subsequently, we found that the Optotracing technology was not limited to the study of biofilms. The same methods to track cellulose in biofilms was transferable to the studying cellulose in plants as shown in Paper 3. Optotracing allowed the visual analysis of material qualities and the kinetics of cellulose digestion. Further characterization of this technology led to Paper 4. This work uncovered the binding site of LCOs and the extraordinary molecular resolution of this technology. Perhaps due to the unique way that they report, LCOs were able to differentiate highly similar polysaccharides that differed only in the α or β configuration.

Being highly interdisciplinary, Papers 2 to 4 has brought up a wide range of topics for future investigation. In particular,

- Do invading pathogens form biofilms in the host?
- How pervalent is cellulose in biofilms of differenct species of bacteria?
- How is curli and cellulose organised in the extracellular matrix?
- What other biofilm components can Optotracing detect?
- Can LCOs be added to a growing plant to view composition changes in cell walls during development.
- Can new Optoracers be synthesized that detects α configured glucose polysaccharides?
- Can we apply one or multiple LCOs to simulataneously track both substrate and products in a process?

As a technology in its infancy, many facets can still be improved. In particular examining optical signatures is not trivial and far from being user friendly. A key direction of future studies will be to simplify and automate data handling.

5 FUTURE PERSPECTIVES: USE-INSPIRED BASIC RESEARCH

Scientific pursuit aims to advance knowledge, by constructing, testing and refining theories. Advances in knowledge in turn supports the next generation of discoveries. This growth model of knowledge necessitates an intimate parallel development of new tools and technologies in order to answer questions of increasing complexity.

My thesis began with Tissue Microbiology, aiming to better understand host-pathogen integrations during infections through intravital studies and highly integrated research approaches. As with most studies, this thesis met with questions that could not be answered by tools available at the time. Consequently, we were compelled to custom develop tools that then enabled the continuation of my thesis, and the discovery of previously unknown facets of the biofilm system. This became a pivotal point of my thesis, triggering a chain in which further refinement tools generated, pursuing the 'range of application' and 'ease of use', expanded the breadth of my research. This generated new fundamental knowledge, and brought to mind the concept of use-inspired basic research. Coined by Donald Stokes, use-inspired basic research lies at the convergence point- when the 'quest for understanding' meets with 'high consideration of use'[232].

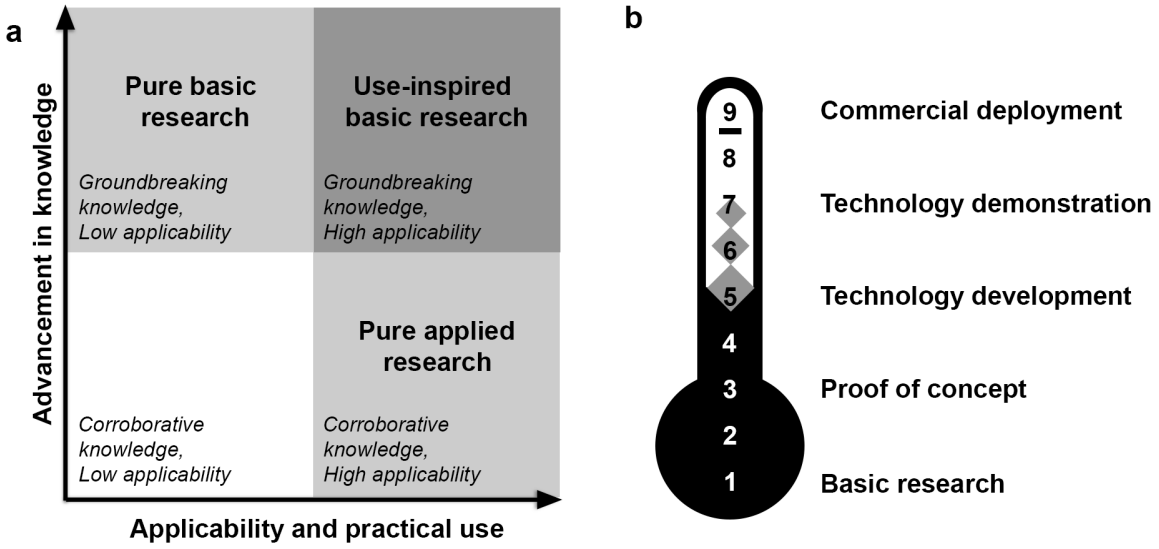

Figure 5: Concept charts guiding this thesis. *(**a**) The Pasteur's quadrant is a classification scheme of research based on the value of the content in advancing scientific knowledge and immediacy of the research for application in society[232]. The defining example of "pasteurization" is ranked in the top right quadrant, representing breakthrough research that solved a key need of society. (**b**) Technology readiness level is a workflow scheme that evaluates the maturity of research/technology for commercial application. This is also a risk-assessment method, to determine the activities necessary for research to transition to product.*

Excellent use-inspired basic research on its own rarely effects on society. Tools that survive the rigors of research to arrive at the pinnacle of Pasteur's quadrant now find themselves at the bottom of the innovation process. At the time of this writing, work done in this thesis has produced a granted patent and an ongoing patent application. From here on, my thesis become heavily influenced by the concept of the "User". This was significant as the distance between basic research to commercial deployment can be staggering depending on the needs of intended "User", as well as the regulatory requirements in governance. It was harrowing to learn that when driving use-inspired basic research to commercials deployment to answer the needs of society, research without a clear definition and vision of the user is merely a hobby. This strongly molded my thesis wherein I began to consider a symbiotic workflow that mutually benefits applicability and academic publications. Coincidentally, as the demand increases for academic research to productively impact society, this may be the approach future scientific pursuits will have to take.

6 POPULAR SCIENCE ABSTRACT

Despite of best efforts, bacterial infections remain one of humanity's greatest challenges. Infections are a major concern in health, healthcare, farming, food production, water and many other aspects of society. Understanding how bacteria live and cause infections requires the right tools to properly study them in their natural environment. Often these tools do not exist and needs to be developed.

A part of this work investigated how the body organizes itself to react to an infection in the kidney. By viewing the infection like a movie using a precise surgery and a special microscope, we identified important 'actors' involved during infection and designed the relevant experiments. In doing so, we found that the body uses nerves, to call to action the spleen. This seems to happen the moment bacteria establish a foothold in the kidney. The spleen then sends out signals through the blood that helps choose the right reactions at the kidney. Using nerves to communicate an infection means that instructions can be sent to all parts of the body instantly, and at the same time, only to the parts that are needed. This is exciting new information that is likely to change the how we think of nerves and the reaction speed of the body to an infection.

Studying infections, we noticed that bacteria tend to live in communities, surrounded by the protective structures they make. However, there was no non-toxic and accurate way to study it in the body. Part of the work here was to develop a new tool to study bacterial communities that was effective and safe for use in the body. To do so, we used LCOs, an exciting group of molecules originally developed for investigating Alzheimer's disease. This created a whole new way of studying bacteria lifestyles. We could now steadily follow and measure each protective structure as the bacteria makes them. This could be done easily, automatically and safely without killing the bacteria. This is important as it meant bacteria research could now be done clearly, with better accuracy, while using less time and materials. The new multi-purposed tool, in a method we call Optotracing, could also be used to study the plants and plant products. Here we used Optotracting to measure and make visible cellulose in a wide range of natural and man made products. Optotracing also allowed us to estimate cellulose amounts in important processes in the industrial and laboratory settings. Due to the unique way this tool works, we were able to generate stunning pictures that showed the location of cellulose in plants and plant based materials like a well-drawn map.

We hope that Optotracing will be used widely in research to both better the understanding of bacteria infections and improve our use of plant resources.

7 MY SCIENTIFIC CONTRIBUTION

Papers presented in this thesis, like the tip of and iceberg, represents a fraction of all work performed over the duration of my post-graduate studies that spanned the fields of man, microbe and plants.

In one aspect, a portion of this thesis contributes to the understanding of how multiple organs in the host is activated and coordinated to respond to a localized and acute infection. Our demonstration of the role of nerves in direct and indirect sensing of UPEC infection via PAMPs, DAMPs, cytokines and chemokines fills an existing knowledge gap and adds to the growing field of nerve-driven immunity.

In another aspect, work done in this thesis resulted in the development of the novel material analysis tool, which we named Optotracing. This was the first demonstration of the trans-disciplinary application of amyloid detection technology developed for diagnosing Alzheimer's disease, to biofilm detection in infection biology, and to Carbohydrate Anatomical Mapping in plants and plant-derived biomasses. As a direct result of my work in this thesis, Optotracing is now maturating rapidly as a commercially available research tool. As an optimist, I believe that the tools and methods developed in my thesis will enable new discoveries in the research of diseases, antimicrobial solutions, bacterial lifestyles, plant development and the better use of plant based resources. At the very least, Optotracing will be a safer, easier, time efficient and sample conserving method for the researcher of today.

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